M 2014

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

UNCOVERING PRMT6 DEREGULATION EFFECT IN PROSTATE CANCER

VÍTOR DIOGO ALMEIDA RIOS
TESE DE MESTRADO APRESENTADA
AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
DA UNIVERSIDADE DO PORTO EM
ONCOLOGIA MOLECULAR

VITOR DIOGO ALMEIDA RIOS

UNCOVERING PRMT6 DEREGULATION EFFECT IN PROSTATE CANCER

Dissertação de mestrado apresentada ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto em Oncologia – Especialização Oncologia Molecular

ORIENTADOR

Professora Doutora Carmen de Lurdes Fonseca Jerónimo

Professora Associada convidada com "Agregação"

Departamento de Patologia e Imunologia Molecular

Instituto de Ciências Biomédicas Abel Salazar Universidade do Porto

Investigadora Auxiliar e coordenadora do Grupo de Epigenética e Biologia do Cancro – Centro de Investigação

Instituto Português de Oncologia – Porto

CO-ORIENTADOR

Professor Doutor Rui Manuel Ferreira Henrique

Professor Catedrático

Departamento de Patologia e Imunologia Molecular

Instituto de Ciências Biomédicas Abel Salazar Universidade do Porto

Diretor do Serviço de Anatomia Patológica

Investigador Sénior do Grupo de Epigenética e Biologia do Cancro – Centro de Investigação

Instituto Português de Oncologia – Porto

"You I advise not to work,
But to fight.
You I advise not to peace,
But to victory.
Let your work be a fight,
Let your peace be a victory."

Friedrich Nietzsche, *Thus Spoke Zarathustra,* 1883

AGRADECIMENTOS

A realização desta tese de Mestrado representou para mim, o culminar de uma etapa importante na minha formação profissional. Há dois anos atrás, a minha ambição centrava-se em adquirir novos conhecimentos numa área do meu interesse e aplicá-los da melhor forma na realização desta tese. Volvidos dois anos, o apaziguamento que sinto mostra-me que esta etapa me enriqueceu muito para além da componente cognitiva e profissional. Desta forma, é legitimo que me sinta grato para com um leque de pessoas que me ajudaram, às quais quero endereçar o meu sincero agradecimento.

À minha orientadora, Prof.ª Carmen Jerónimo, em primeiro lugar porque me mostrou que a lealdade é base essencial para respeitar e ser respeitado. Numa fase menos positiva para mim, ter acreditado no meu trabalho foi um gesto que me motivou até à última palavra desta tese. Penso que a cada contratempo que encontrei ao longo deste caminho, o desânimo que não conseguia esconder espelhava suficientemente bem a vontade que tinha em retribuir o voto de confiança em mim depositado. Nesses momentos menos conseguidos, o seu lado maternal foi essencial em cada conselho que me deu, em cada lição que me incutiu. A maneira singular como gere o Grupo de Epigenética e Biologia do Cancro ofereceu-me liberdade para seguir um determinado rumo de trabalho e para aprender com os erros cometidos.

Ao meu Co-orientador, Prof. Rui Henrique, cujo vasto conhecimento, rigor científico e espírito crítico apenas são excedidos pela simplicidade e educação com que trata todos aqueles que o procuram. Talvez tenha sido abusivo da minha parte procurá-lo tantas vezes para discutir resultados e esclarecer dúvidas, mas teria sido ingénuo em desperdiçar a oportunidade de trabalhar e aprender, com alguém tão competente para partilhar o conhecimento como o Professor.

A ambos, expresso o meu singelo agradecimento e considero-me um privilegiado por ter tido a oportunidade de trabalhar e aprender convosco. A este e a tantos outros níveis, são e serão no futuro, uma referência para mim.

Ao Prof. Manuel Teixeira, Diretor do Serviço de Genética e do Centro de Investigação do Instituto Português de Oncologia do Porto, por me ter aceite no mesmo e me ter dado todas as condições necessárias para a elaboração deste trabalho.

À Filipa, em primeiro lugar pela dura batalha que travou para que não a tratasse por "Professora Filipa". Alcançada essa vitória, foi a pessoa que me acolheu no âmbito laboratorial quando eu ainda tinha um conhecimento muito superficial do que era estar num laboratório de investigação. Ensinaste-me tudo aquilo que necessitei para conseguir ser autónomo e orgulho-me de sentir que, neste sentido, tive os melhores ensinamentos possíveis. Se hoje me sinto metódico e organizado naquilo que faço, devo-o a ti. As risadas e os ombros que partilhámos neste período são a prova que fomos mais que companheiros de trabalho. Fomos fomentando uma boa amizade que, sem ser de sempre, espero que seja para sempre.

À Inês, porque em boa hora apareceste nesta caminhada. Esse sorriso de orelha a orelha é a melhor recordação que posso guardar dos bons momentos partilhados. Foste um pilar essencial no rumo que este trabalho levou. Quando, numa fase decisiva, sofri um enorme revés, passível de colocar em causa a realização de um bom trabalho, tu foste absolutamente fantástica na forma como suportaste o meu mau humor, me motivaste e prontamente te disponibilizaste para me ajudar naquilo que precisasse. A forma empenhada e séria como discutiste os resultados obtidos e deste sugestões pertinentes, foram sem dúvida alguma, cruciais na etapa final e decisiva desta tese. Conciliar tudo isto com boa disposição constante, fez com que o tempo que trabalhámos juntos e facilitámos o difícil, seja uma imagem a guardar deste percurso.

À Márcia, porque por motivos profissionais partilhámos cada dia deste percurso. Aprendemos a lidar com os nossos dias bons, os nossos dias maus e a tolerar a falta de sincronização entre os nossos dias bons e os nossos dias maus. Não foi fácil, nem tão pouco difícil. Foi uma experiência nossa. Cresci a cada desavença, a cada debate e a cada partilha de ideias. Por tudo isto, que os rumos que as nossas vidas levem, me façam sentir a tua ausência e que a ausência traga até mim a memória de cada riso incontrolado, soltado por razões que só nós conseguíamos perceber.

Ao Pedro, à Joana e ao Diogo, por toda a partilha de ideias e ajuda que sempre me deram ao longo deste trabalho. Ao Pedro, porque te prestaste a ajudar quando precisei de ti e pelo rigor e espírito crítico que te caracterizam. À Joana porque sempre que te procurei e precisei de ti, foste simplesmente impecável. Ao Diogo porque bastavam uns minutos a falar contigo para a minha

cultura geral sofrer um *upgrade*. Embora eu não saiba o que vou levar desta vida, espero conseguir levar a memória. Nela guardo todos os bons momentos que passámos, desde os lanches aos jantares e desde os jantares ao jogos no Dragão a sofrer pelo nosso Porto. Que no futuro nos possamos encontrar e que o reencontro seja nostálgico por aquilo que nos marcou, a boa disposição.

Ao João, porque de uma forma profissional e particular foi discutindo comigo os resultados que fui obtendo, sugerindo sempre possíveis linhas de trabalho a seguir. Agradeço-te a partilha de ideias e de *standards* pois permitiram enriquecer o conteúdo desta tese.

A todos os elementos do Grupo de Epigenética e Biologia do Cancro que conheci nestes dois anos. A vossa capacidade de conciliar o trabalho com a boa disposição é admirável e, sem qualquer sombra de dúvida, trabalhar assim torna-se bem mais fácil.

À Francisca, por todas as conversas científicas que tivemos e que me deixaram a pensar em teorias mirabolantes. Devo ainda um agradecimento especial às tuas aptidões para a alquimia, pois quase me mostraste que era possível tornar o preto, branco.

A todos os meus colegas do Mestrado em Oncologia, pela boa experiência social que foi conhecer-vos, adquirir conhecimentos em conjunto e partilhar a ansiedade que antecedeu cada exame ou trabalho coletivo.

Ao Jorge Sousa, porque chegar a casa e abstrair-me do trabalho nunca foi fácil. A cada tentativa de te explicar o que andava a fazer, estou certo que te enriqueci tanto cientificamente como tu a mim musicalmente, de cada vez que tive de ouvir o teu repertório para saxofone, desde a nobre música clássica à irreverente música contemporânea.

A todos os elementos da minha equipa de futebol, assim como da adversária, que todas as segundas-feiras permitiram pausar o trabalho com uma agradável hora de competição saudável. Até nas derrotas foi uma honra partilhar as quatros linhas convosco. Que o nosso companheirismo continue a ser o nosso lema e o símbolo máximo da camisa que envergamos.

À Técnica Maria do Amparo, que no decurso deste período me permitiu disponibilizar o tempo necessário para não comprometer a execução desta tese.

Foi uma boa experiência para cada um de nós e no final desta etapa, reconheço com gratidão a compreensão para com a minha situação. O rigor e o profissionalismo que desde cedo me incutiu foram pedra basilar para fortalecer o meu sentido de responsabilidade, pelo que hoje lhe devo este gesto. Como tantas vezes me disse, eu ainda sou "uma criança com muito para aprender e crescer", mas terminada esta etapa, cada conselho que me deu, foi a base para que hoje consiga dar passos mais sólidos neste processo de crescimento.

À Dr.ª Mariana, porque apesar da "indolência do pai", sempre me conseguiu transmitir o seu afecto de uma forma bastante peculiar. Não sou muito astuto na forma como lido com combustíveis e afins, mas em compensação, a pureza e simplicidade que senti em cada gesto teu, foram o combustível eficaz para impulsionar os bons momentos que recordo. As certeiras sugestões para distrair do trabalho, os divertidos jantares a três que tantas gargalhadas nos fizeram soltar.. Que esta fase tenha sido o início de uma longa e linda amizade.

Ao Luís Leça, porque com tantas razões para te agradecer, a grande dificuldade é escolher as melhores. É difícil encontrar uma justificação para entender o que é que nos tornou tão próximos. Independentemente do que tenha sido, foi sólido o suficiente para que te sinta hoje como família. Somos dois irmãos que o destino quis encontrar. Começamos por partilhar um campo de futebol sem bancadas e hoje partilhamos um estádio cheio de bons momentos. Que a nossa amizade seja como as pautas da música que fazemos, sempre numa tonalidade maior e com a interpretação que nos apetecer dar. Porque foi neste tom que nos tornámos dois verdadeiros amigos e será assim que esta amizade permanecerá intemporal.

Ao Romeu, por todas as visitas que me fizeste. Foi um prazer ser recebido ao portão por alguém que mostra tanto a saudade como a sente. Vou continuar a querer chegar a casa e ter de dar a tua voltinha de popó, seguida da sessão de pugilismo no teu brinquedo favorito.

Às minhas irmãs, Silvana e Margarida, porque foram um apoio seguro em cada fim de semana passado em família. Despropositadamente mas de uma forma eficiente, a vossa boa disposição foi a melhor distração depois de uma semana de trabalho. Sei que não lidam bem com as saudades do vosso irmão, mas pensem sempre que aquilo que nos une é bem maior que a distância que nos separa. ☺

Por último, aos verdadeiros pilares de tudo isto, os meus pais. Seria demasiado redutor dizer que vocês foram maravilhosos nestes dois anos, pois já o são desde há 24 anos atrás. A educação e os princípios que me incutiram são a razão primordial daquilo que sou hoje. A forma incansável como me deram tudo o que precisava para conseguir atingir as minhas ambições, é algo que jamais esquecerei e eternamente valorizarei. É em vocês que eu me revejo e é na vossa maior obra, a nossa família, que eu encontro tudo o que preciso para me sentir seguro. E como as palavras começam a ficar um meio limitado para expressar o quanto eu vos estou grato, fico-me por vos dizer que vocês são quem eu mais quero que sintam esta vitória comigo. Esta e todas as que nos esperam. Esta Dissertação de mestrado é dedicada a vós.

A todos vós, o meu sincero e profundo agradecimento.

RESUMO

O carcinoma da próstata (CaP) é, mundialmente, uma das neoplasias malignas com maior incidência e prevalência no sexo masculino, constituindo uma das principais causas de morbilidade e mortalidade oncológica. O conhecimento limitado da sua biologia compromete o desenvolvimento de novos marcadores de diagnóstico e prognóstico, capazes de melhorar a monitorização e terapêutica desta neoplasia. Atualmente é reconhecido que a desregulação epigenética desempenha um papel fundamental na carcinogénese prostática. A expressão anormal de enzimas modificadoras de histonas, nomeadamente das metiltransferases (MTHs) e das desmetilases (DMHs), tem sido associada ao desenvolvimento do CaP, bem como as modificações pós-transducionais (MPTs) das histonas estabelecidas por estas enzinas. Contudo, a importância da desregulação da atividade/expressão de alguns membros das MTHs e DMHs permanece ainda por esclarecer.

Num estudo prévio usando uma amostragem reduzida, verificámos que a MTH PRMT6 estava significativamente sobrexpressa no CaP comparativamente aos tecidos prostáticos normais (TPNs), tendo igualmente demonstrado uma boa capacidade na distinção entre TPNs e CaP. Tendo como base estes resultados preliminares, os principais objectivos desta dissertação de mestrado foram validar a sobrexpressão da PRMT6 em CaP numa série alargada, bem como investigar o seu putativo papel oncogénico na carcinogénese prostática, recorrendo a modelos *in vitro.*

A sobrexpressão da PRMT6 foi observada quer ao nível do transcrito quer ao nível da sua expressão proteica tanto nos CaP como nos PIN.

Os estudos *in vitro* na linha celular PC-3 silenciada (PC-3 Sh-PRMT6) demonstraram um aumento significativo das células em apoptose, assim como a diminuição da viabilidade celular e da capacidade migratória e invasiva. Contrariamente, na linha celular LNCaP o silenciamento da PRMT6 não atenuou o fenótipo maligno, provavelmente, devido ao seu *status* de expressão de receptores de androgénios. Adicionalmente, a nível molecular a linha celular PC-3 Sh-PRMT6 apresentou diminuição global na expressão da marca H3R2me2a. Contudo, não se verificou alteração nos padrões de expressão da marca H3K4me3, embora os níveis de expressão das enzimas catalisadoras desta MPT

(complexo MLL e a SMYD3) tenham aumentado significativamente. O silenciamento da PRMT6 associou-se significativamente com o aumento dos níveis de transcrito do p21, p27 e CD44, bem como com a diminuição da expressão da MMP-9. Relativamente às vias de sinalização celular, o silenciamento da PRMT6 conduziu a uma redução da via PI3K/AKT/mTOR e a um aumento da via dos receptores de androgénios, sugerindo um papel oncogénico desta enzima.

De modo a confirmar que as alterações observadas são uma consequência direta da atividade da PRMT6 serão necessários estudos complementares. De igual modo, serão efetuados ensaios adicionais *in vitro* para esclarecer a utilidade terapêutica da re-expressão dos receptores de androgénios na linha celular PC-3. De facto, a restauração da expressão do AR em células PC-3 Sh-PRMT6, pode ser clinicamente relevante, uma vez que pode voltar a sensibilizar células neoplásicas andrógeno-insensitive a ADT.

SUMMARY

Prostate cancer (PCa) is one of the most incident and prevalent cancers in men worldwide, and a leading cause of cancer-related morbidity and mortality. The limited knowledge about its biology hinders the development of new diagnostic and prognostic markers, able to improve management and therapeutic of this malignancy. Epigenetics plays an important role in prostate carcinogenesis and in fact, abnormal expression of histone modifier enzymes, such as histone methyltransferases (HMTs) and demethylases (HDMs), and its chromatin modifications are related to PCa. Notwithstanding, the specific role of deregulated activity/expression of several members of both HMTs and HDMs is still poorly understood.

Previously, we have found in a small number of clinical samples that PRMT6 was overexpressed in PCa compared to normal prostate tissue samples (NPTs) and demonstrated a promising capacity to distinguish NPTs from PCa. Based on these previous results, the main goals of this work were to validate the overexpression of PRMT6 in PCa and further explore its putative oncogenic role in prostate carcinogenesis, using *in vitro* models.

Using a large series of prostatic samples we found that PRMT6 was overexpressed in PCa, at both transcript and protein level. Intriguingly, PIN lesions displayed significantly higher PRMT6 expression levels, compared to PCa. Furthermore, PRMT6 mRNA levels are able to discriminate cancerous from non-cancerous prostate tissues.

Contrarily to LNCaP, stable PRMT6 knockdown in PC-3, attenuated the malignant phenotype, in which the increased apoptosis as well as the decreased viability levels, migration and invasion ability was observed.

Moreover, at molecular level, PRMT6 silencing was associated with decreased H3R2me2a levels and increased MLL complex and SMYD3 expression, although no global H3K4me3 levels were found.

The silencing of PRMT6 significantly associated with increased expression levels of p21, p27 and CD44, as well as decreased expression of MMP-9. Regarding signaling pathways, PRMT6 knockdown related with a reduction of the PI3K / AKT / mTOR pathway and an increase in AR, supporting an oncogenic role for this enzyme.

Nevertheless, to confirm the observed alterations as a direct consequence of PRMT6 activity further experiments are needed. Similarly, additional *in vitro* studies should be performed to elucidate the specific therapeutic utility of AR re-expression in PC-3 cell line. Indeed, restoration of AR expression in Sh-PRMT6 PC-3 cells, might be of clinical relevance as it may re-sensitize androgen-insensitive neoplastic cells to ADT.

TABLE OF CONTENTS

FIGURE INDEX

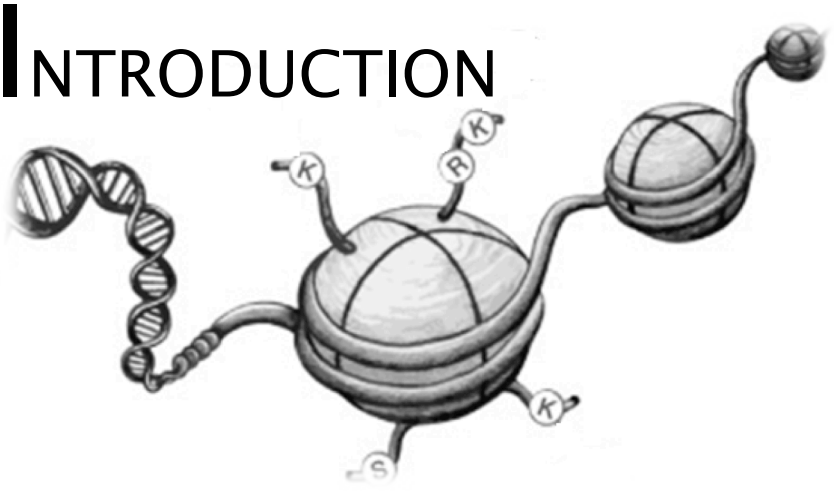
TABLE INDEX

LIST OF ABBREVIATIONS

ADT	Androgen Deprivation Therapy
AR	Androgen Receptor
AS	Active Surveillance
BPH	Benign Prostatic Hyperplasia
BSA	Bovine serum albumin
CRPC	Castration-Resistant Prostate Cancer
DRE	Digital Rectal Examination
EZH2	Enhancer of Zeste Homolog 2
FBS	Fetal bovine serum
GS	Gleason Score
GUSB	Glucuronidase, Beta
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HGPIN	High-grade PIN
HMT	Histone Methyltransferase or Histone Methylase
ISUP	International Society of Urological Pathology
JMJC	Jumonji C
KDM	Lysine-Specific demethylase
KMT	Lysine histone methyltransferase or lysine histone methylase
LSD	Lysine specific demethylase
miRNAs	MicroRNAs
MLL	Mixed-Lineage Leukemia
MRI	Magnetic Resonance Imaging
ncRNA	Non-Coding RNAs
NPT	Normal Prostate Tissue
NSD1	Nuclear Receptor Binding SET Domain Protein 1
PCa	Prostate cancer
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia

PRMT	Protein Arginine Methyltransferase
PSA	Prostate Specific Antigen
PTEN	Phosphatase and tensin homolog
PTMs	Post-Translational Modifications
RP	Radical Prostatectomy
SAM	S-adenosyl-methionine
SMYD3	SET And MYND Domain Containing 3
TRUS	Transrectal Ultrasound
UICC	Union for International Cancer Control
WHO	World Health Organization

INTRODUCTION



1 PROSTATE

1.1 Prostate Anatomy and Histology

Herophilus of Alexandria first used the term "prostate" in 335 b.C., referring the organ localized in front of human urinary bladder [1]. The human prostate is a pear-shaped exocrine gland and as part of the male reproductive system plays an essential role in secretion of important components of the seminal fluid, as well as in ejaculation [2-4] .The prostate base is located at the bladder neck and its apex at the urogenital diaphragm [5, 6]. In normal adults, prostate weights up to 20 g and measures approximately 25cm^3 [2, 4].

In 1981, McNeal proposed the most widely accepted model of prostate anatomic organization (Figure 1) [7]. According to McNeal, prostate is divided in four basic regions: peripheral, central and transition zones and anterior fibromuscular stroma (AFMS) (Figure 1). The peripheral zone constitutes about 70% of the glandular prostate and is located at the posterior and lateral sides of the gland. The central zone represents about 25% of the glandular tissue, it is posterior to the urethra and surrounds the two ejaculatory ducts. The transitional zone encases the prostatic urethra and accounts for 5% of glandular elements. AFMS forms the entire anterior surface of the prostate as a thick non-glandular layer, which extends from the bladder neck to the striated sphincter [6-8].

Histologically, acini and ducts compose the prostate´s glandular component. The ducts are subdivided into large (primary, major or excretory) and peripheral (secondary or minor). Typically, both acini and ducts contain two layers of epithelial cells: secretory cells and basal cells [2]. Moreover, neuroendocrine cells are rare in normal prostate epithelium [4].

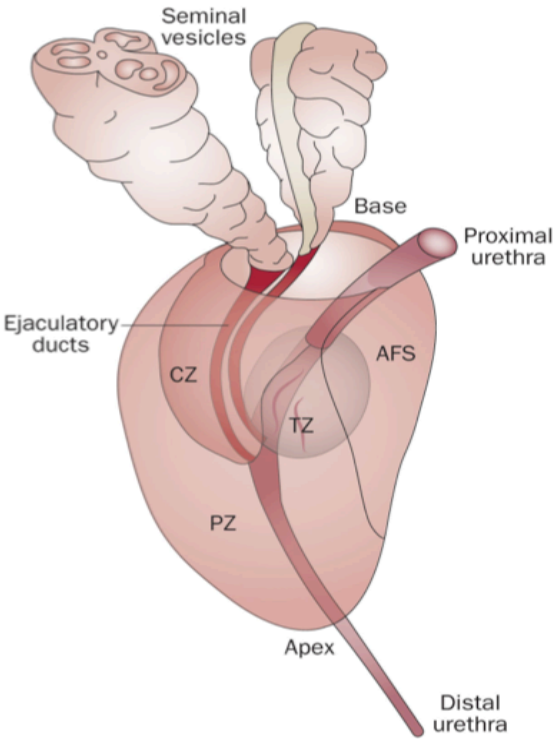


Figure 1: Anterior oblique view of the prostate and urethra. The prostate is divided into four zones: central zone (CZ), transition zone (TZ), peripheral zone (PZ) and anterior fibromuscular stroma (AFS). Adapted from [8].

1.2 Non-malignant Disorders of Prostate

Prostate has higher frequency and extent of non-malignant lesions than other organs [9], being more common in aged men [10]. Benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) are among the most common pre-malignant lesions of this gland.

BPH is a chronic disease affecting more than half of the male population over 50 years old [10]. Coexistence of BPH and prostate cancer (PCa) was first described in 1957 in a necropsy study, in which BPH was identified in 80% and 45% of cadavers with and without PCa, respectively [11]. Although BHP and PCa share several common pathophysiological mechanisms they exhibit important differences in terms of histology and localization. However, it is still not clear if BHP represents the first step in the pathway to PCa [11].

In 1987, **PIN** lesions were first described by Bostwick and Brawer [12] being those lesion classified into low-grade (LGPIN) and high-grade (HGPIN). Although, only HGPIN is widely accepted as a precursor lesion of PCa.

This lesion is characterized by progressive phenotype and genotype abnormalities that are intermediate between benign prostatic epithelium and cancer [13, 14]. The frequency of HGPIN in prostate biopsies averages 9% and the severity and frequency of these lesions at *postmortem* examination are greatly increased in individuals with cancerous prostates comparing with noncancerous prostates [13, 15]. In addition to the similar cytologic features, HGPIN and PCa share genetic and molecular markers as well as other characteristics, such increased incidence with age and preferential occurrence at prostatic peripheral zone. These characteristics, combined with the fact that HGPIN precedes the onset of PCa in about 10 years, suggest that HGPIN represents an intermediate stage between benign epithelium and the invasive malignant carcinoma. Thus, HGPIN might have a clinical significance as a tool to identify patients at risk of malignancy [14].

PIA has been also suggested as a precursor of PCa and HGPIN. This lesion is described as a hyperproliferative entity associated with chronic inflammation and with distinct morphological appearance recognized as prostatic atrophy [16]. The hypothesis that PIA could be a potential precursor of other prostate lesions stems from the fact that this lesion usually occurs in the prostate peripheral zone and shares some molecular features found both in PIN and PCa [13]. Despite those findings, evidence to date is inconclusive as to whether PIA is a precursor of HGPIN and PCa [13].

1.3 Prostate Cancer

Adenocarcinoma of the prostate accounts for 95% of all malignancies in this gland [3]. PCa is an age-related disease, being very heterogeneous in terms of pathologic, biologic and clinical behavior, ranging from clinical indolent to highly aggressive neoplasia [6, 17]. This malignancy is multicentric and has no specific symptoms, which leads to a late diagnosis and compromises not only prognosis but also the attempts to develop target therapies [6].

Histologically, PCa is characterized by loss of both basal cell layer and membrane. Remarkably, in HGPIN this cell layer is fragmented and in BPH, both are intact. This finding suggests the existence of a sequential pathway in PCa development (Figure 2) although the underlying mechanisms that lead to PCa still not fully understood [18].

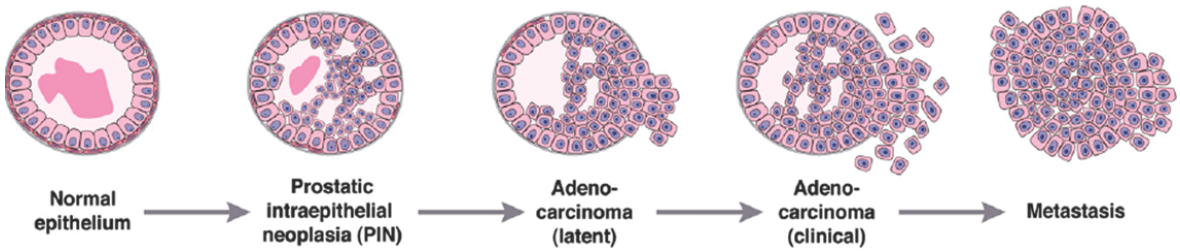


Figure 2: Progression pathway for prostate cancer. Adapted from [3].

1.3.1 Epidemiology of Prostate Cancer

Current epidemiological data indicates this neoplasm as a major health concern. In 2012, 1,111,689 new cases were diagnosed worldwide (approximately 15% of total newly diagnosed cancers in men), making it the second most commonly diagnosed cancer in male population, only outshined by lung cancer [19].

PCa incidence rates are very heterogeneous around globe, being estimated more than a 25-fold variation. In fact, about 70% of new diagnosed cases in 2012 occurred in more developed countries, being the higher incidences reported in United States, Australia and Nordic countries, whereas lower incidence rates are observed in Asia [19]. (Figure 3)

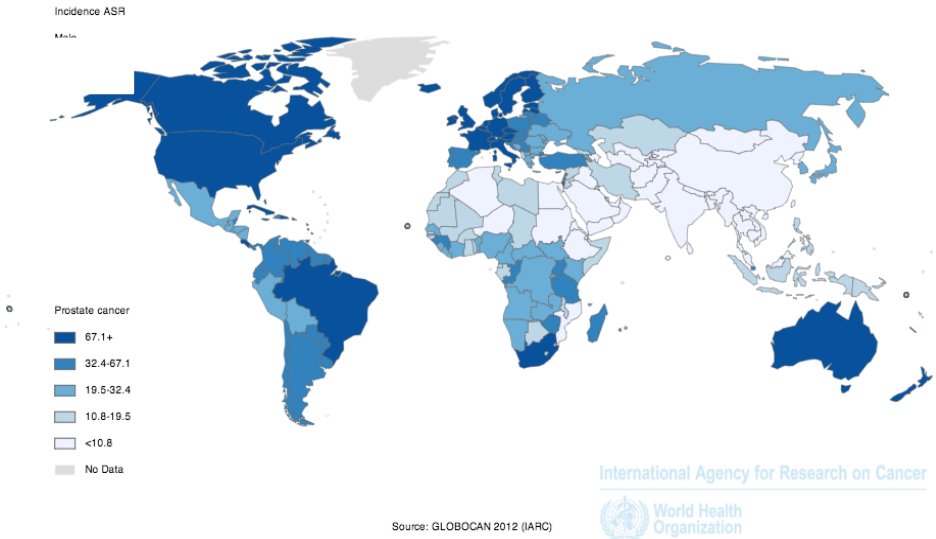


Figure 3: Estimated age-standardized incidence rates of PCa per 100,000, worldwide. Adapted from [19].

Regarding mortality, a slight decrease in mortality rates were observed among developed countries. However, the mortality rates are much less variable than incidence, accounting only a 10-fold variation worldwide [19].

Restricting this analysis to Europe, PCa arises as the most incident cancer and the third cause of cancer-related mortality. In Portugal, PCa is also the most frequent cancer among men, with 6,622 newly diagnosed cases in 2012 (23,3% of overall cancers) and the third leading cause of cancer related death (11,1% of overall cancer related deaths) [19]. (Figure 4)

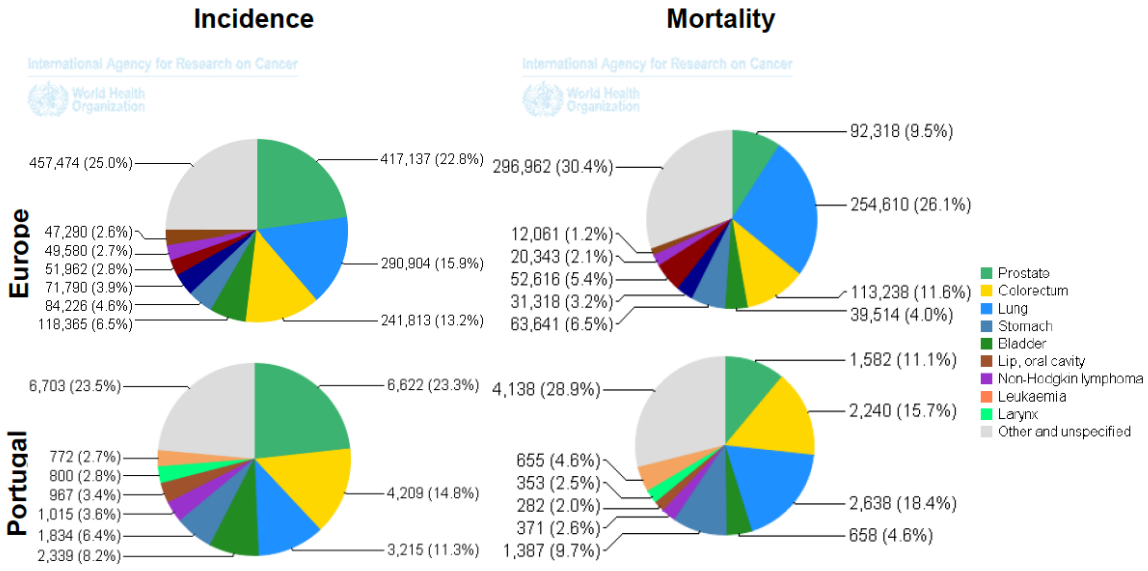


Figure 4: Incidence and mortality of different cancers in Europe and Portugal. Adapted from [19].

1.3.2 Risk Factors

Although the etiology and pathogenesis of PCa is quite complex, there are three well-established and widely accepted risk factors [20].

Race: PCa remains as one of the most singular cancers regarding geographical distribution. Globally, African American men have a higher risk to develop PCa and tend to be diagnosed at younger ages and with more advanced stage, which reflects the higher mortality rates in this population, comparing to European American men [21].

Asian men have the lowest incidence rates of PCa but Asian males living in the United States exhibit a higher risk to develop this malignancy [22]. The exact and scientific explanation for these disparities is still unknown, however genetic,

environment, social or cultural factors also in addition to differences in the decision-making practice, access to medical care and preferred diagnostic tools, might play an important role in this unequal distribution of PCa around the globe [23, 24].

Family history: Men´s probability of developing PCa is higher within a family history of the disease is present. Currently, it is estimated that 10-15% of all PCa are associated with familial cases [23]. In fact, men with first-degree relatives (father or brother) harboring PCa have more than double of risk to develop this disease. Additionally, the number of members affected and the early-onset of disease increase even more the risk of developing PCa. This might suggest an involvement of a genetic predisposition, although other features like exposure to the same environmental factors should also be considered [22, 23].

Age: As a consequence of a clinical latent course, age is the most important recognized risk factor for PCa incidence [17, 23]. Malignancies of prostate gland are more frequent in older men, with a median age at diagnosis of 67 years. Approximately 85% of PCa cases are diagnosed after the age of 65 years old and in men older than 90 years old, the incidence is higher than 90% [22, 23, 25].

1.3.3 Current Guidelines for Prostate Cancer Diagnosis

Diagnosis of PCa is quite hampered by indolent behavior of this malignancy. Thus, improvement of detection tools to diagnose the disease when it is organ confined is the best approach. Serum concentration of prostate-specific antigen (PSA) and digital rectal examination (DRE) are generally used as a standard methodology to PCa screening and management [26]. DRE is capable to detect about 18% of all PCa, regardless of the PSA level [27].

PSA is a glycoprotein with protease activity produced by epithelial cells of prostate gland, which is secreted in the glands lumina and is released into the bloodstream in increased quantities when disruption of normal prostatic membrane structures occurs. This phenomenon arises not only in malignant lesions, but also in the context of inflammation, hyperplasia and urologic manipulation. Therefore, PSA is prostate specific, but not PCa specific. Additionally, PSA not only lacks sensitivity to detect an important number of PCa

cases, especially at early stages, but also increases the overdiagnosis and overtreatment of indolent tumors, demonstrated a limited value as a screening test [28].

According with international guidelines, men with inconclusive or suspicious DRE result and/or PSA value equal or higher than 4.0 ng/mL, must be considered for transrectal ultrasound (TRUS)-guided biopsy. Additionally, when a patient is considered for biopsy, clinicians should take into account factors such patients' age, possible comorbidities and therapeutic consequences. It should be taken in consideration that both PSA and DRE are fallible, once there are PCa cases with normal DRE and/or lower levels of PSA [29-31].

Nevertheless, the combination of PSA test and DRE allowed for an important decrease in the number of cases with advanced disease at the diagnosis (about 70-80% of PCa are organ confined), which increases the disease-free survival [4].

*1.3.4 **P**rostate **C**ancer **G**rading **S**ystem: **G**leason **S**core*

The Gleason score (GS) is the widespread accepted system for grading PCa. This system is based on the histological evaluation of glandular epithelial architecture pattern, excluding cytological aspects from the analysis. Given the PCa heterogeneity, it classifies the two most prevalent patterns in a 1 to 5 scale, with a decreasing grade of differentiation (1- Most differentiated; 5- Least differentiated) (Figure 5). Thus, the GS consists in the sum of the classification of the two most common patterns, ranging from 2 (1+1) to 10 (5+5) [4, 32].

In 2005, International Society of Urological Pathology (ISUP) published the last update of this classification, considering that GS of a prostate biopsy should include the Gleason grade of the prevalent carcinoma pattern and the highest grade, independently of its extent. The predominant pattern has a crucial role in prognostic evaluation, specifically in GS = 7 lesions, because the predominance of pattern 4 (Gleason 4+3) carries more than a 3-fold higher risk of PCa mortality than Gleason pattern 3+4 [33, 34].

Since 1966, when Donald Gleason firstly proposed this grading system, it is along with pathologic stage, a powerful tool for PCa prognostic assessment. Thus, GS is fundamental in predicting PCa natural history and the risk of recurrence after radical prostatectomy (RP) or radiotherapy, influencing treatment approach [30].

Notwithstanding, inter-observer variability remains the principal pitfall of GS, although the updated system has proved to increase in 20% agreement between observers [32, 33].

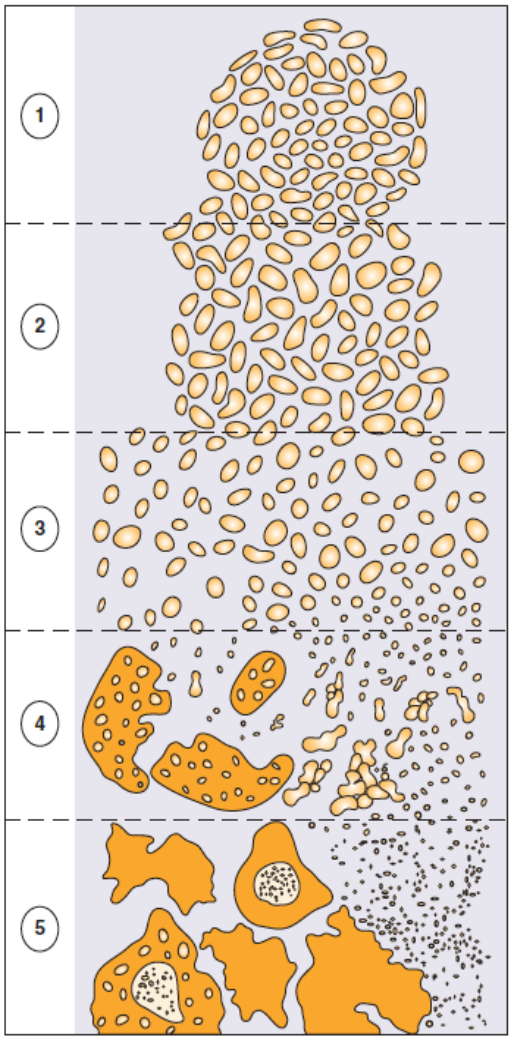


Figure 5: Modified Gleason grading system for PCa.
Pattern 1: Closely-packed, uniform, rounded to oval glands;
Pattern 2: More loosely arranged glands with smooth ends that minimally invade non neoplastic tissue;
Pattern 3: Irregular size and shape glands with more infiltrative margins;
Pattern 4: Fused, cribriform or ill-defined glands;
Pattern 5: Almost no glandular differentiation. Adapted from [34].

*1.3.5 **C**linical and **P**athological **S**taging*

PCa is an important cause of cancer morbidity and mortality worldwide and therefore, accurate staging is a crucial approach for prognosis assessment and treatment planning [35].

The most commonly used staging system for PCa was proposed by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC): the TNM (Tumor Node Metastasis) system [36]. This staging system consists in the evaluation of primary tumor (T) extension, regional involved lymph nodes (N) and presence or absence of distant metastases (M) (Table 1) [37]. According with AJCC and UICC, TNM stage for PCa could be clinical (cTNM) or pathological (pTNM) [38].

Clinical stage provides information regarding cancer extension, before definitive treatment onset and is generally achieved by DRE, TRUS or magnetic resonance imaging (MRI), and serum PSA levels [36, 39].

Since pathological staging requires macro and microscopic evaluation of surgical specimen and dissected regional lymph nodes, it can only be determined after RP [4, 38, 40]. Consensually, pathological staging predicts recurrence with more accuracy than clinical stage and, it is also useful as a prognostic factor [40]. In this sense, clinicians developed monograms, combining independent prognostic factors such as capsule invasion, preoperative serum PSA levels, GS for the RP specimen, positive surgical margins, lymph node metastases, seminal vesicles involvement and distant metastases [4, 35, 41].

Table 1: The 2010 AJCC/UICC TNM staging classification for PCa. Adapted from [36].

PRIMARY TUMOR (T)	
	CLINICAL
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent tumor neither palpable nor visible by imaging
T1a	Tumor incidental histologic finding in 5% or less of tumor resected
T1b	Tumor incidental histologic finding in more than 5% of tumor resected
T1c	Tumor identified by needle biopsy
T2	Tumor confined within prostate gland
T2a	Tumor involves one half of one side or less
T2b	Tumor involves more than one half of one lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through prostate capsule
T3a	Extracapsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as: external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
	PATHOLOGIC (PT)*
pT2	Organ confined
pT2a	Unilateral, one half of one side or less
pT2b	Unilateral, involving more than one half of one lobe but not both lobes
pT2c	Bilateral disease
pT3	Extraprostatic extension
pT3a	Extraprostatic extension or microscopic invasion of bladder neck
pT3b	Seminal vesicle invasion
pT4	Invasion of rectum, levator muscles and/or pelvic wall
REGIONAL LYMPH NODES (N)	
	CLINICAL
Nx	Regional lymph nodes were not assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
	PATHOLOGIC (PN)
pNx	Regional nodes not sampled
pN0	No positive regional nodes
pN1	Metastasis in regional node(s)
DISTANT METASTASIS (M)	
M0	No distant metastasis
M1	Distant metastasis
M1a	Non-regional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease

*There is no pathologic pT1 classification

*1.3.6 **P**rostate **C**ancer **T**herapies*

Unequivocally, PCa is associated with a substantial mortality and morbidity, but in fact, most men do die with, rather than of, their PCa. Furthermore, given the intrinsic heterogeneity of PCa, men with similar history (clinical stage, serum PSA levels and biopsy features) can have significantly different outcomes. Hereupon, the main challenge to physicians is to recognize the faction of men with aggressive local PCa and thus choose a suitable therapy approach for these, sparing the remainder of the morbidity associated to overtreatment [4]. Current therapeutic options for PCa include surgery, radiation, hormone therapy and chemotherapy. Treatment choice usually depends on disease and patient ´s features such as age, life expectancy, life quality, TNM stage, GS and preoperative serum PSA level [37].

Concerning **clinically localized PCa**, standard treatment options are active surveillance (AS), RP, external-beam radiotherapy and interstitial radiation therapy (brachytherapy).

AS is suitable for patients with very-low risk of PCa progression. The requirements for belong to this group of patients are: T1-T2 TNM staging (organ confined PCa), GS ≤6 and serum PSA level < 10ng/mL. These patients are subjected to frequent PSA measurements and prostatic biopsies to detect disease progression [31].

RP remains the standard procedure for high-risk locally advanced PCa, showing survival benefits comparing with conservative methodologies. Patients with a biopsy GS ≤8, TNM staging ≤ T3a, PSA level < 20ng/mL and life expectancy of 10 or more years are indicated for this surgery. This procedure consists in the removal of whole prostate gland and seminal vesicles and has been optimized in order to decrease the associated morbidities, such as erectile dysfunction and urinary incontinence [31, 42].

Other therapeutic option for organ-confined PCa is radiotherapy since both external-beam radiotherapy and brachytherapy present rates of disease-free survival similar to that obtained by RP. External-beam radiotherapy is a non-invasive methodology with low morbidity rates suitable for patients without disseminated disease and which not tolerate RP or brachytherapy [42]. Regarding brachytherapy, this approach consists in the permanent ultrasound guided insertion of radioactive seeds, with the half-life of 60 days, directly in the

prostate. This methodology has fewer side effects when compared to RP and external-beam radiotherapy, being recommended for patients with low-volume and low-grade PCa and [42, 43]. A combination of these two modalities of radiotherapy improves survival outcome and is used in patients harboring intermediate to high-risk PCa [4].

Considering **high risk PCa**, hormone therapy (i.e. androgen deprivation therapy [ADT]) is the widely accepted therapeutic strategy. Once prostate is a hormone-responsive gland and about 80-90% of PCa are sensitive to AR action at the time of diagnosis, hormone therapy aims to avoid androgen effect either by suppressing its secretion or inhibiting its action [42, 44].

Generally, ADT consists in administration of Gonadotropin-releasing hormone analogs (GnRH), also called luteinizing hormone releasing-hormone (LHRH), concomitantly with anti-androgens (i.e. flutamide and bicalutamide), causing a significant reduction of prostatic secretory cells by apoptosis [3, 45, 46]. Additionally, ADT can also be used as neoadjuvant or adjuvant therapy, in combination with surgical or radiation therapy [47].

Even if ADT has a palliative intent and, indeed, leads to 70-80% symptoms reduction, within 18 to 24 months from treatment initiation most tumors acquire a hormone refractory phenotype, also known as castration-resistant disease. [48-50].

Regarding **castration-resistant PCa (CRPC)**, this stage of PCa is often connected with biochemical progression (increased levels of serum PSA), as well as by metastatic progression [51]. In order to understand the progression to CPRC, two models have been proposed: the adaptation model and clonal selection model (Figure 6) [51, 52].

The adaptation model supports that androgen-dependent PCa cells experience genetic and epigenetic events that allow themselves adapt to androgen-depleted environment, culminating in ADT resistance that consequently leads to cancer relapse. The mechanisms that confer resistance to ADT include increase of androgen levels in tumor, amplification of *AR* gene, gain-of-fuction mutations, alternative-splice variants of AR, changes in expression of co-regulatory molecules, androgen independent activation of AR signaling and other bypass pathways that enables cancer development, under the pressure of androgen-deprivation conditions [51].

Conversely, clonal selection model proposes that subclones of PCa cells with different degrees of androgen dependence are already present in tumor before onset of ADT and the selective pressure of low-androgen conditions leads to the outgrowth of the pre-existing castration-resistant clones [51].

In this last stage of PCa, the morbidity and mortality rates are significantly higher and chemotherapy (docetaxel combined with either prednisone or estramustine, the first-line treatment in metastatic CRPC) is one of the few available options, exhibiting a limited efficiency (response rate about 10-20%) and short survival (only 2 months) [48-50, 53]. Mitoxantrone combined with prednisone is the second-line approved treatment for metastatic CRPC, being capable to improve patients' life quality (reduction of pain), however it does not show a survival benefit [54, 55].

Indeed, considering that metastatic CRPC is incurable, the available treatments only aim to improve patients' life quality.

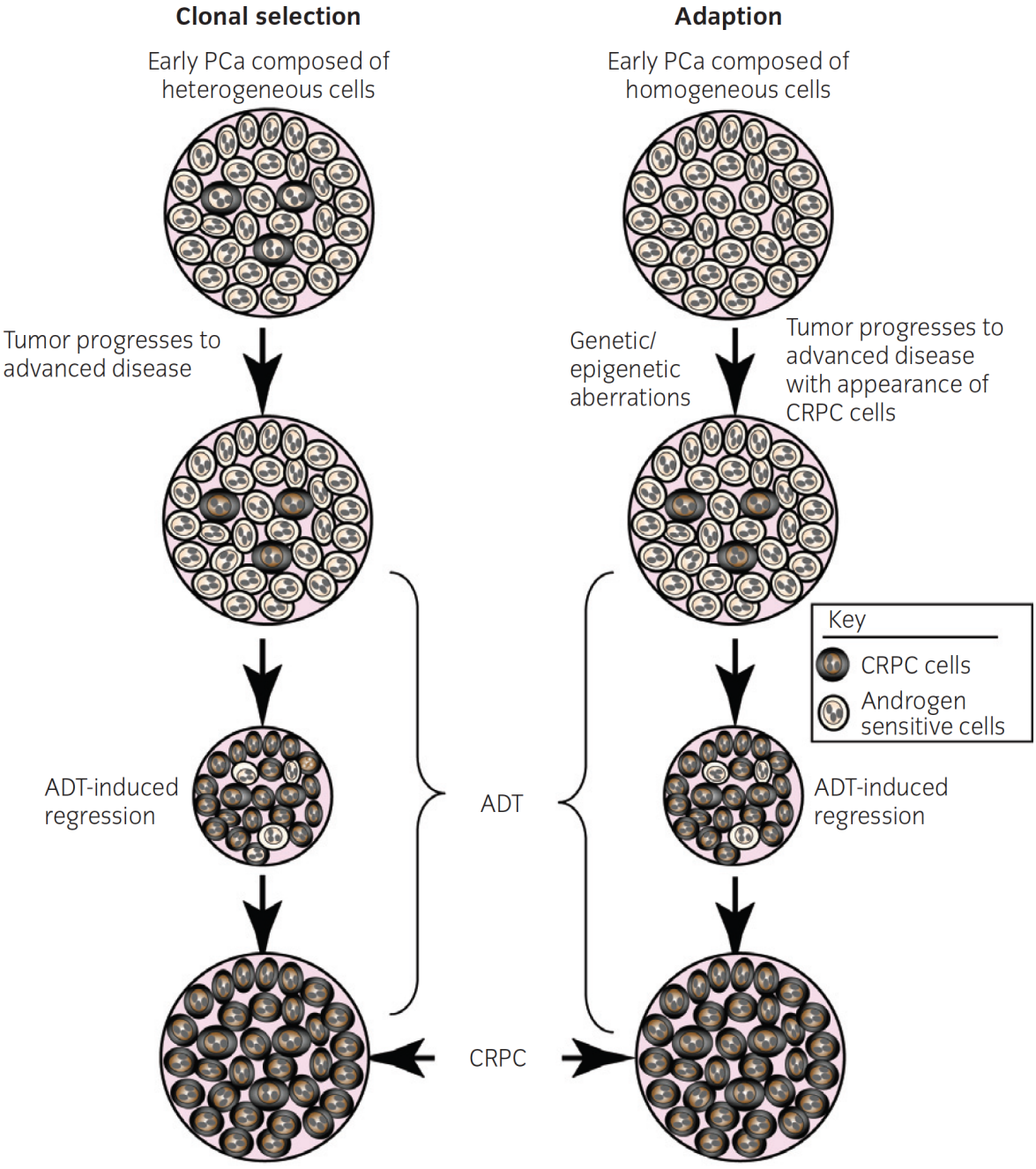


Figure 6: Models of progression to CRPC. Adapted from [52].

2 EPIGENETICS

2.1 Deciphering its Growing Relevance

The paradigmatic question about what exactly we are and how we evolve over the years is no more answered that we are only the reflection of the genetic code that composes our DNA. In a precise and accurate manner we can find this answer in Lehninger´s classic textbook: we are our proteins. In fact, proteins are the final point of the central dogma of molecular biology, since they are generated from our DNA and this process is intermediated by RNA molecules. However, not all RNA molecules encode proteins, although these non-coding molecules have crucial cellular functions. So we are not just our genes, they are solely part of the history and this is where epigenetics takes its role [56].

Etymologically, "Epigenetics" derives from the Greek *epi* - which means beyond – genetics. Conrad Waddington firstly used this term to define "the casual interactions between genes and their products, which bring the phenotype into being" [57]. Actually, epigenetic defines heritable and transient changes in gene expression that are not due to any alterations in the DNA sequence [58]. This emerging field provides the scientific basis to explain differences between two monozygotic twins, on two cloned animals artificially generated from the same DNA donor, the expression of only one X chromosome in female or simply the fact that just paternal or maternal traits are expressed in offspring [56, 59-61].

The three major epigenetics mechanisms are: DNA methylation, histone post-translation modifications and non-coding RNAs. This highly sophisticated machinery is crucial to regulate chromatin structure and transcriptional activity [62]. Epigenetics marks are primarily established during embryonic development and play a crucial role throughout differentiation, being maintained across during cellular replication and division [62, 63].

Despite being an area in expansion, epigenetic show a promisor evolutional capacity as Esteller clearly described: "*Genetics has had several jump-starts since the late recognition of Mendel's work, the elucidation of the double-strand DNA structure by Watson, Crick, Wilkins, and Franklin, and the completion of the human genome projects led by Francis Collins and Craig Venter. In epigenetics, we are still trying to catch up. What lies ahead could be even more exciting.*" [56].

2.2 Epigenetic Mechanisms of Gene Expression Control

2.2.1 DNA Methylation

Among the three epigenetic mechanisms of gene expression control previously mentioned, DNA methylation is, so far, the most studied [64]. Briefly, DNA methylation consists in the addition of a methyl group at the 5' position of a cytosine ring inside CpG dinucleotides being this enzymatic reaction carried out by DNA methyltransferases [65]. CpG dinucleotides are distributed in an organized manner throughout the genome, with condensing of these dinucleotides at 5'-gene regulatory region, recognized as CpG islands [66]. Interestingly, a great portion of human gene promoters present a CpG island, which are frequently unmethylated in normal cells and become specifically methylated during development and tissue differentiation [64].

Regarding transcriptional impact, DNA methylation is associated with chromatin repressive states and consequently with inhibition of gene expression [64]. This repressive effect of DNA methylation can be exerted by directly avoiding RNA polymerase and transcription factors binding [65, 67] or indirectly, through the recruitment of methyl-CpG-binding domain (MBD) proteins which in turn recruits histone-modifying and chromatin-remodeling complexes [64].

DNA methylation patterns are found altered in disease processes, especially in cancer [68]. Generally tumors show a global decrease in DNA methylation levels, specifically in repetitive DNA sequences, coding regions and introns, leading to a progressive malignant phenotype through events such as chromosomal instability, reactivation of transposable elements and loss of imprinting [57]. Simultaneously and with the same carcinogenic contribute, also occurs hypermethylation at specific CpG islands, mainly in promoter regions of tumor-suppressor genes [57, 64, 69] (Figure 7).

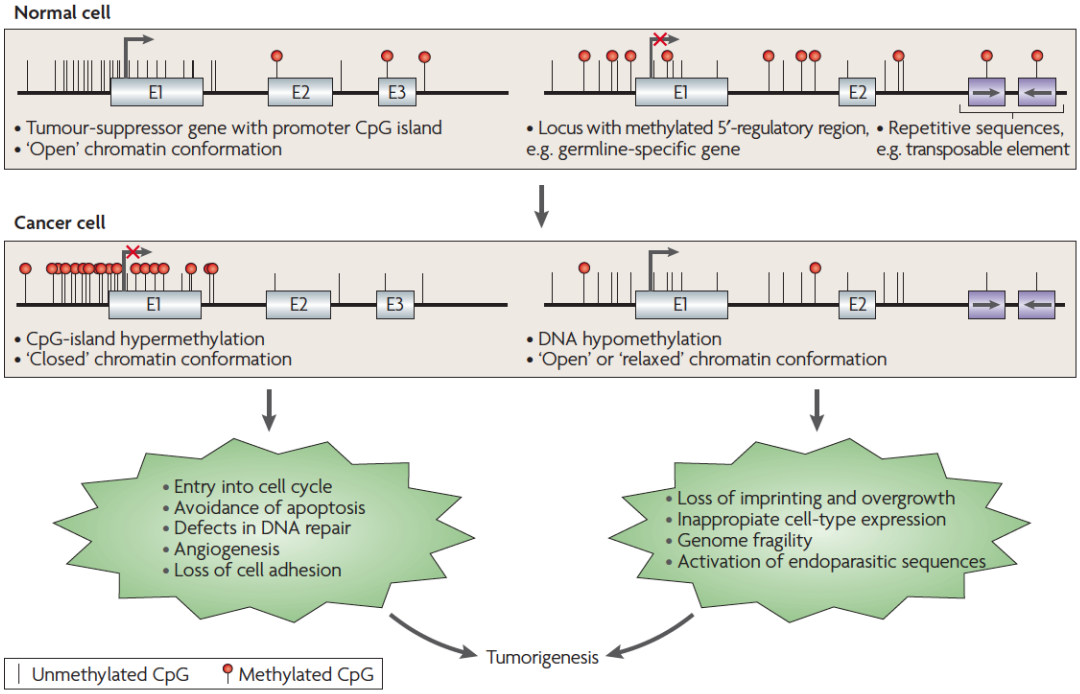


Figure 7: Altered DNA-methylation patterns in carcinogenesis. The hypermethylation of CpG islands of TSGs is a common alteration in cancer cells leading to the transcriptional inactivation of these genes and the loss of their normal cellular functions. In cancer cells, is observed a global hypomethylation, especially at repetitive sequences. Adapted from [69].

2.2.2 *Non-coding RNAs*

In the last decade, attention has been focused in non-coding RNAs (ncRNAs). In fact, only 2% of human genome codifies proteins and about 90% is transcribed into RNA molecules [70, 71]. Although these molecules do not encode proteins, they are very important in specific cellular pathways such as splicing, chromosome dynamics, RNA editing, inhibition of translation, mRNA destruction, X-chromosome silencing in females and DNA imprinting [72, 73]. Depending on interactions and activity, ncRNAs are divided in: small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), transcribed ultraconserved regions (T-UCRs), large intergenic non-coding RNAs (lincRNAs), long non-coding RNAs (lncRNAs) and the spotlight within the group, microRNAs (miRNAs) [74].

MicroRNAs (miRNAs), the most studied class of ncRNAs, repress the mRNA translation either by degradation or inhibition of mRNA, depending on the accuracy of matching miRNA-mRNA [75, 76]. Even so, although several studies describe miRNAs as repressors of transcription, recent data have indicated that they could also act as positive regulators of transcription [77]. This small size class of ncRNAs composes only about 1% of human genome, however it's

estimated that they regulate the expression of 50% of human genes [78]. Additionally, one miRNA can target many mRNAs, which in turn can be targeted by several miRNAs [75]. Thereby, due the scope of miRNA regulation, they play important functions in biological processes such as apoptosis, differentiation and proliferation [78, 79].

MiRNAs aberrant expression has been widely reported in several diseases, especially in cancer. The disruption of normal miRNA expression patterns is associated with genetic and epigenetic events [71, 74, 80]. A trend to a global miRNAs downregulation is observed in cancer, although upregulation also has been described [81]. Concerning miRNA role in carcinogenesis, depending on the neoplastic context and target genes, miRNAs can act as oncogenes (oncomiRs) or tumor-suppressors [82]. Hereupon, tumor-suppressors miRNAs decreased expression in cancer can be attributed to deficiencies in miRNA biogenesis leading to anomalous expression of their targets. In turn, oncomiRs overexpression and/or amplification promote a decreased expression of their miRNA-target tumor-suppressor genes, which could contribute to tumor induction [82].

*2.2.3 **H**istone **P**ost-**T**ranslational **M**odifications*

In eukaryotic cells, chromatin is the macromolecular complex formed by DNA and histone proteins that contains the inherited material of cells [83]. The basic functional unit of chromatin, nucleosomes, contain 147 base pairs of DNA wrapped around a histone octamer, which is composed by one pair of histones H2A, H2B, H3 and H4, linked by H1 (Figure 8) [83-85]. A great portion of histone covalent post-translational modifications (PTMs) is established in histone tails [86]. PTMs define the chemical modifications of proteins that signal to other proteins, which in turn recognize the modification [87]. Additionally, chromatin is a dynamic structure that is divided in two major regions: heterochromatin, which is recognized as highly condensed regions that contain inactive genes; and euchromatin, which is relatively open and comprise transcriptional active genes [83]. There are several PTMs described so far such as methylation, acetylation, phosphorylation, ubiquitylation, sumoylation and deamination.

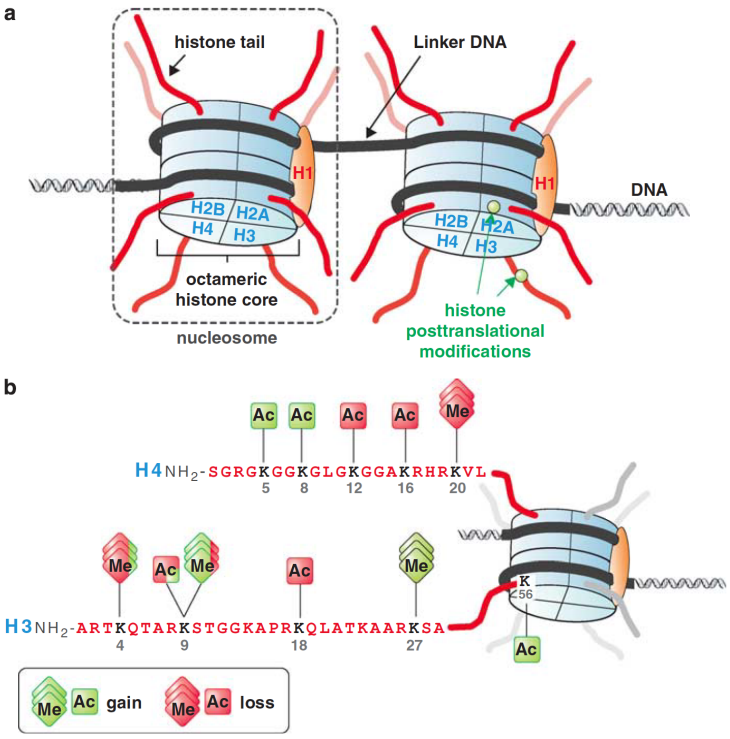


Figure 8: Chromatin structure and histone PTMs. (a) Chromatin is made of repeating units of nucleosomes, composed by ≈ 147 base pairs of DNA wrapped around a histone octamer consisting in two copies each of the core histones H2A, H2B, H3 and H4. Linker H1 is positioned on top of the nucleosome core. (b) Histone PTMs on histone tails (N-terminal). Adapted from [85].

The epigenetic machinery responsible for the dynamics of PTMs can be divided into different groups based on their biological function: writers that lay down epigenetic marks in histones; readers which recognize them; and erasers that remove the PTMs (Figure 9) [88]. The distinct patterns of PTMs within the histone tails have been recognized as "histone code" and along with DNA methylation play a major role in gene activity regulation [83, 89]

Regarding methylation and acetylation, the respective epigenetic writers comprise histone methyltransferases (HMTs) and histone acetyltransferases (HATs), while epigenetic erasers are composed by histone demethylases (HDMs) and histone deacetylases (HDACs) [88].

Histone acetylation has an important impact in gene transcriptional activity since it affects the electrostatic charge of the histones. Alterations in electrostatic charge of the histones influence not only the chromatin structure but also the binding affinity of histones to DNA. Indeed, the chromatin packaging controls the capacity of epigenetic readers to recognize histone PTMs in histone tails and thus, active or repress transcription [88, 90, 91].

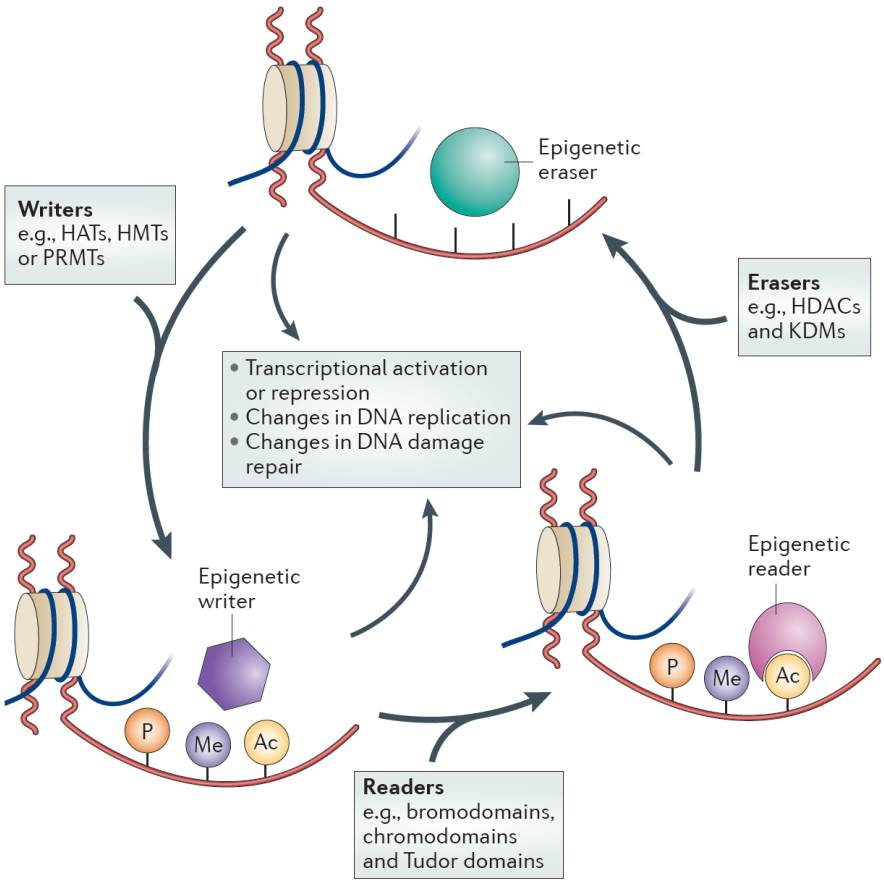


Figure 9: Epigenetic writers, readers and erasers. Adapted from [88].

Since histone PTMs are crucial for gene transcription regulation, they require a balance between the epigenetic machinery responsible by their dynamics. Disruption of this balanced is associated with pathological processes including cancer [92]. However, the plasticity nature of PTMs allowed the developing of "epigenetic drugs", capable to target abnormal activity/expression of important chromatin-modifying enzymes [93, 94].

3 HISTONE METHYLATION AND PROSTATE CANCER

3.1 Histone Methylation: Basics of an Extensive Field

Unlike acetylation, histone methylation does not alter the charge of the target residue, whereby is unlikely to be able to directly change chromatin structure. Methylation marks serves as binding sites for proteins that maintain nucleosomes together or signalizes for additional regulatory proteins such as binding domains [87]. Histone methylation occurs mainly in lysine residues (K), by lysine methyltransferases (KMTs), and in arginine residues (R) by arginine methyltransferases (PRMTs). Lysines can be mono- (me1), di- (me2) or tri-methylated (me3), whereas arginines might be mono- and symmetrically (me2s) or asymmetrically di-methylated (me2a) [93].

Transcriptional effect of histone methylation is relatively more complex than histone acetylation. Histone methylation can either lead to transcription repression or gene activation depending on the specific target residue, as well as the degree of methylation (Figure 10) [95].

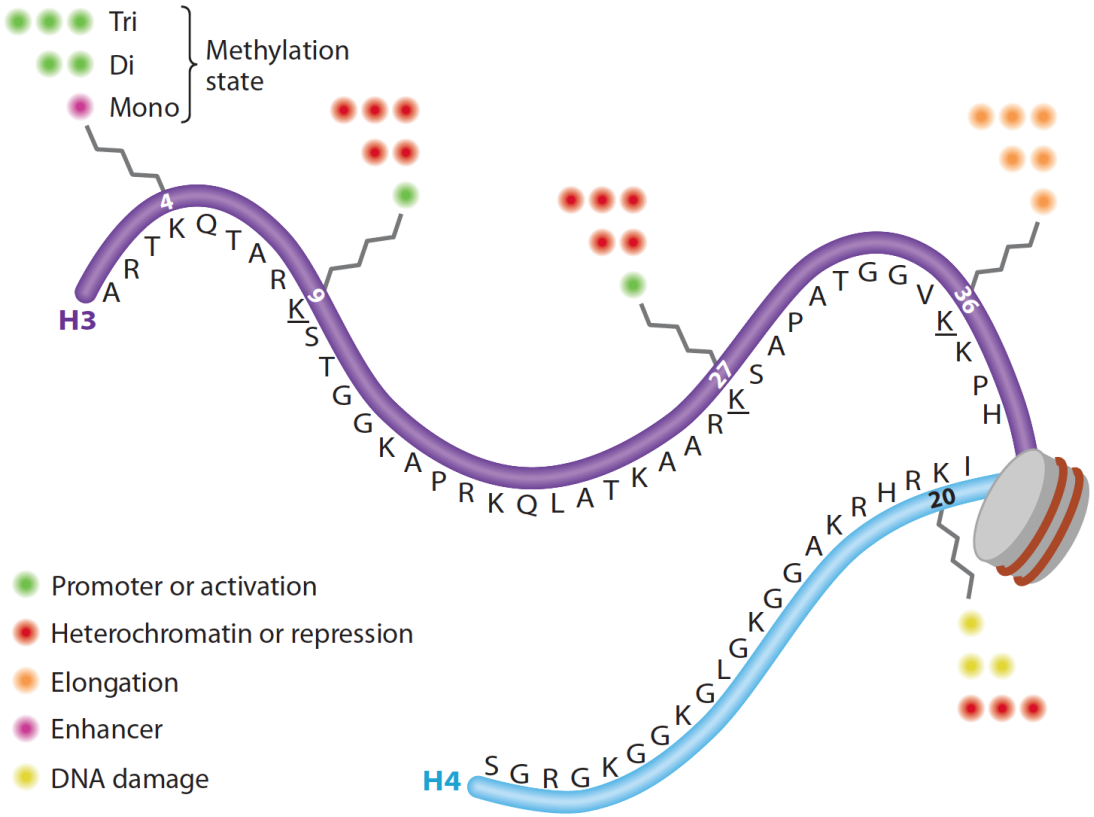


Figure 10: Major lysine methylation marks on the amino-termini of histones H3 and H4. The embedded numbers refer to the methylated amino acid residue on each histone. The general function of each mono- , di- and trimethylation state is depicted in dots of distinct colors, according with its function. Adapted from [95].

In actively transcribed genes, there is an enrichment of H3K4me2 and H3K4me3, H3K36me3 and H3K79. Conversely, H3K9me3, H3K27me3 and H4K20me3 are associated with repressed genes [96]. More than 50 HMTs have been described, so far, and all of them use S-adenosylmethionine (SAM) as a co-substrate to transfer methyl groups [87, 93]. Additionally, HMTs can be classified in three different groups: SET domain KMTs, non-SET domain KMTs and PRMTs [97]. Regarding PRMTs, they might be classified in three types: Type I, II and III generate monomethylarginine (MMA). Specifically, type I catalyzes the subsequent generation of asymmetric dimethylarginine (ADMA) and type II catalyzes symmetric dimethylarginine (SDMA) [98]. Histone methylation was considered for many years a stable and static histone modification until the discovery of the first KDM (Lysine-specific demethylase 1 - LSD1) in 2004 [93]. Since then, more than 30 HDMs were described and classified in two families: Lys-specific demethylases (LSD) and jumonji C (JMJC) histone demethylases [96]. This dynamic character of histone methylation requires an efficient balance between HMTs and HDMs in order to avoid alterations in normal cellular phenotype [92].

3.2 **H**istone **M**ethylation: **R**elevance in **P**rostate **C**ancer

Aberrant histone modification profiles as well as the deregulated activity of correspondent enzymes are linked to cancer onset and progression, mainly by two mechanisms: alterations on normal gene expression of both oncogenes and TSGs, and genomic instability [93] (Figure 11).

Concerning PCa, several alterations of normal histone modifications patterns have been associated with different clinical outcomes. While H3K4me1 and H3K4me2 are associated with increased risk of PCa recurrence [99, 100], methylation of H3K4 and H3K27 was correlated with tumor grade or recurrence [99, 101]. Additionally, levels of H4R3me2 and H3K4me2 allowed the distinction between two groups of low-grade PCa with different prognosis outcome (GS = 6 or GS < 6) [102].

Similarly to aberrant patterns of histone modifications, abnormal expression of some histone modifying-enzymes was also reported in PCa. Deregulation of some KMTs, such as Mixed-Lineage Leukemia 2 (MLL2), Mixed-Lineage Leukemia 3 (MLL3), nuclear receptor binding SET domain protein 1 (NSD1), enhancer of zeste homolog 2 (EZH2) or SET and MYND domain containing

3 (SMYD3), were already described in PCa [99, 103]. EZH2 catalyzes repressive marks (H3K27me3 and at less extent H3K9me2) and was demonstrated to be a driver in prostatic carcinogenesis [102, 104-107]. EZH2 was found overexpressed in metastatic PCa being associated with intensification of tumoral phenotype [108, 109].

Moreover, repression of AR target genes was correlated with methylation of H3K9. Some studies showed that knockdown of its specific demethylases (LSD1, lysine-specific demethylase 3A – KDM3A; lysine-specific demethylase 4C – KDM4C) increased the levels of repressive marks on the regulatory regions of AR targeted genes, which in turn leads to their decreased expression [110, 111]. Additionally, LSD1 upregulation is associated with aggressive PCa, CRPC and high risk of disease relapse [112-114].

Therefore, a better understanding of the mechanisms underlying disruption of methylation marks in cancer is needed to develop new drugs and molecular strategies, capable to inhibit the imbalance between HMTs and HDMs, responsible for aberrant deposition of histone´s methylation marks.

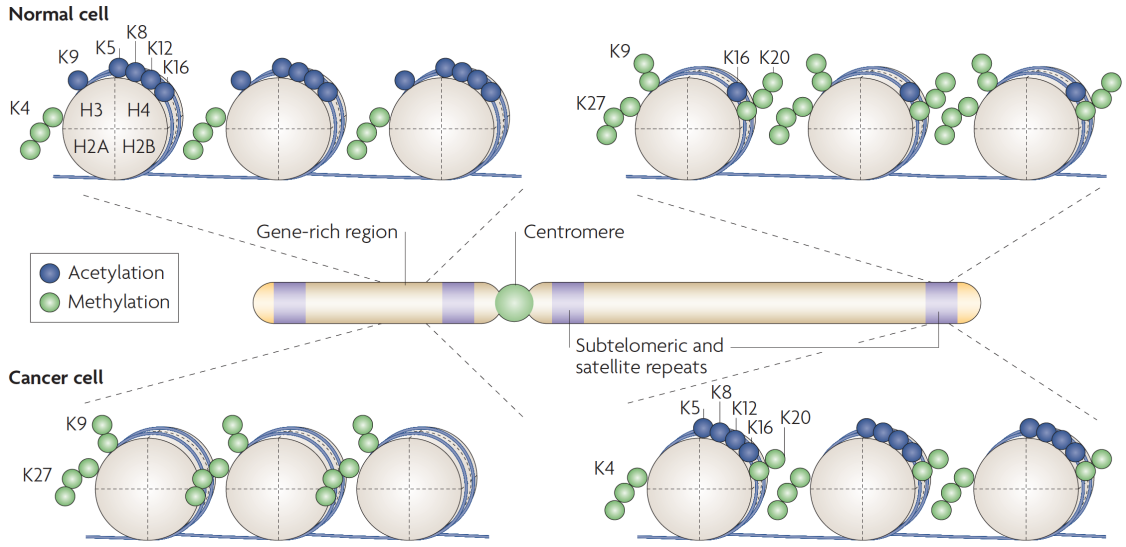
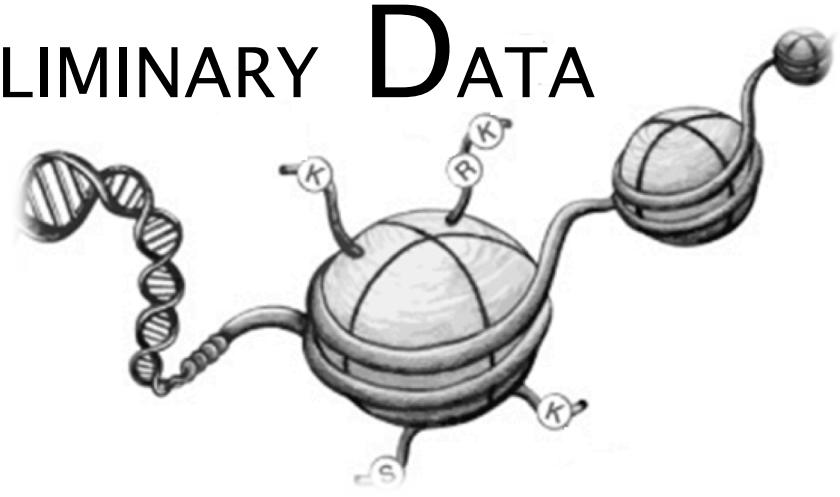


Figure 11: Histone-modifications map for a typical chromosome in normal and cancer cell. In normal cell, histone marks associated with active transcription are enriched in genomic regions including the promoters of tumor-suppressor genes, whereas DNA repeats and other heterochromatic regions are characterized by presence of repressive marks. In cancer cells, there are loss of the 'active' histone marks on tumor-suppressor gene promoters, and loss of repressive marks at subtelomeric DNA and other DNA repeats, leading to a more 'relaxed' chromatin conformation in these regions. Adapted from [69].

Preliminary Data



1 BRIEF CONTEXTUALIZATION

The work presented in this Master Thesis arises from a previous PhD project developed in Cancer Epigenetics and Biology Group (GBEC), which aimed to clarify the role of HMTs and HDMs in PCa.

Thereby, in order to identify what HMTs and HDMs displayed an abnormal expression pattern during prostatic carcinogenesis, TaqMan Array 96-Well Plates were designed to assess expression levels on 37 HMTs and 20 HDMs in normal prostate and PCa tissue samples by RT-qPCR.

Five normal prostate tissues (NPTs) and ten PCa samples were selected to cover the full spectrum of prostate carcinomas considering the GS and pathological stage (relevant clinical and pathological data are depicted in Table 2).

The mRNA levels of the genes studied were normalized to the beta-glucuronidase (GUSB) reference gene and the median value of NPTs and PCa samples was chosen to calculate the fold variation in gene expression between groups, using the comparative *C*t method.

Table 2: Clinical and pathological features of patients included in the screening of HMTs and HMDs expression in NPTs and PCa samples.

CLINICOPATHOLOGICAL FEATURES	PCA	NPT
NUMBER OF PATIENTS, N	10	5
AGE (YEARS)		
Median (range)	59 (53 – 71)	61 (49 – 66)
PSA LEVELS (NG/ML)		
Median (range)	12.3 (3.5 – 19.9)	NA
PATHOLOGICAL STAGE, N (%)		
pT2	4 (40.0)	NA
pT3a	2 (20.0)	
pT3b	4 (40.0)	
GLEASON SCORE, N (%)		
< 7	3 (30.0)	NA
≥ 7	7 (70.0)	

NA, Not applicable

The result of this screening of HMTs and HDMs expression levels showed an overexpression of protein arginine methyltransferase 6 (PRMT6) in PCa samples comparing with NPTs samples (Figure 12).

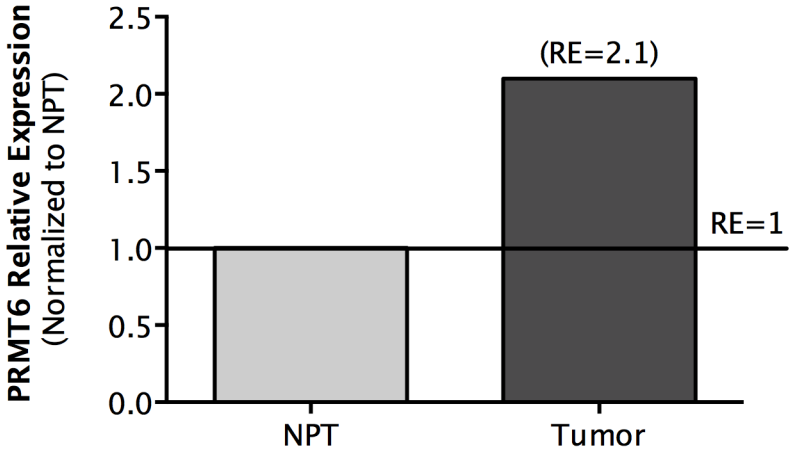
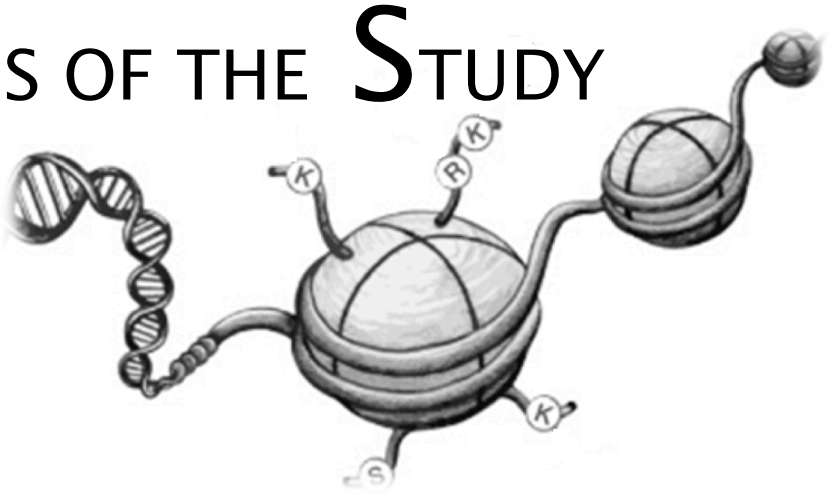


Figure 12: Expression levels of PRMT6 in NPT and Tumor.

Aims of the Study



1 AIMS

PCa is one of the most incident cancers in men worldwide. Mortality rates are not decreasing and their prevalence shows a worrying rising trend to the next years. This malignancy is quite complex and heterogeneous, showing a clinical course that ranges from indolent tumors to highly aggressive and metastatic ones. Additionally, therapeutic options for early-stage PCa exhibit good outcomes but in advanced stages, treatment is far less effective.

Therefore, the major clinical challenge is to identify PCa cases with highly specific and sensitive methods; and concomitantly, to discriminate men with aggressive disease to better adequate treatment option and avoid overtreatment and unnecessary morbidities in patients with clinically indolent tumors.

Histone PTMs are thought to be involved in the control of gene expression either by influencing chromatin compaction and signaling to several protein complexes. Hence, an appropriate expression of the enzymatic machinery responsible for dynamic regulation of histone PTMs is required for appropriate gene expression. Furthermore, abnormal HMTs and HDMs expression patterns as well as the altered patterns of histone PTMs have been found associated with carcinogenesis.

As previously mentioned, PRMT6 was found upregulated in PCa samples when compared to NPT samples. Additionally, this arginine methyltransferase was also found upregulated in other cancers, such as lung and bladder cancer [115].

Hereupon, the main purpose of this Master Thesis was to further explore the importance of PRMT6 deregulation in prostate carcinogenesis. In order to achieve this objective, this project was comprised in the following set of tasks:

I. Confirmation of the overexpression of PRMT6 in PCa, in an independent and relatively large series of prostatic tissue (NPT, HBP, PIN and PCa samples);

II. To ascertain the correlation between PRMT6 expression with clinicopathological parameters;

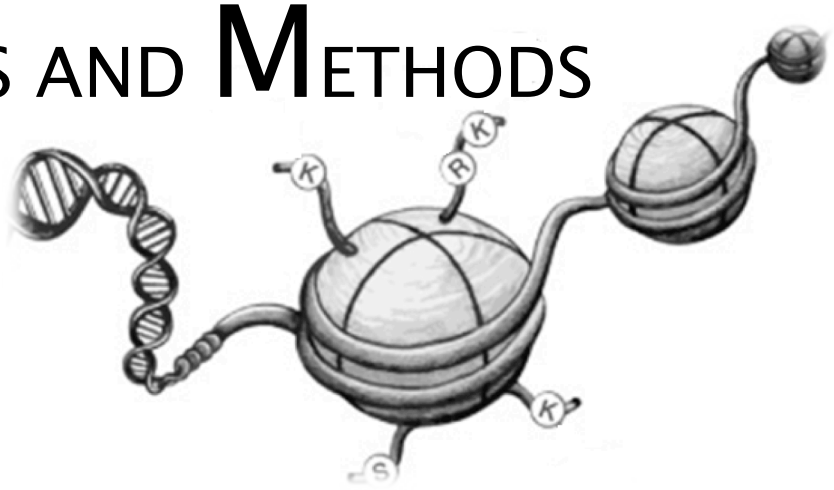
III. Assess the expression of PRMT6, at protein level, in the same series of prostatic tissue used in the first task;

IV. Evaluate the impact of PRMT6 knockdown in malignant phenotype of PCa cell lines;

V. Verify the effect of PRMT6 knockdown in histone PTMs´ patterns;

VI. Identify putative target genes and molecular pathways regulated by PRMT6;

Materials and Methods



1 CLINICAL SAMPLES: PATIENTS AND SAMPLE COLLECTION

Samples of 195 primary tumors of prostate and 38 PIN lesions were prospectively collected from patients with clinically localized prostate adenocarcinoma, consecutively diagnosed and primarily treated with RP at the Portuguese Oncology Institute, Porto, Portugal. For control purposes, samples of 15 non-neoplastic prostate tissues normal prostate tissue (NPT) were obtained from the peripheral zone of prostates not harboring PCa, collected from cystoprostatectomy specimens of bladder cancer patients. All tissue specimens were immediately frozen after surgery and storage at -80ºC, following informed consent. Five-micron thick frozen sections were cut and stained for the identification of the areas of PCa (*i.e.*, the index or dominant tumor) and normal tissue. Then, the tissue block was trimmed to maximize the yield of target cells (>70% of target cells). Histological slides from formalin-fixed, paraffin embedded (FFPE) tissue fragments were also obtained from the same surgical specimens and assessed for GS and TNM stage. Relevant clinical data were collected from the clinical records, and these studies were approved by the institutional review board [Comissão de Ética para a Saúde-(CES 295/2013)] of Portuguese Oncology Institute - Porto, Portugal.

2 RNA ISOLATION

All tissue samples were suspended in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and total RNA was purified using the PureLink™ _ RNA Mini Kit (Invitrogen) following manufacturer's recommendations. The concentration, purity and integrity of RNA samples were determined on a Nanodrop ND-1000 (ThermoScientific, Wilmington, DE, USA) and agarose gel electrophoresis and then RNA samples were stored ate -80ºC.

3 *PRMT6* GENE EXPRESSION IN CLINICAL SAMPLES

PRMT6 mRNA levels were assessed in the previously described series of prostate tissue samples used in this study. Briefly, a total of 300ng was reverse

transcribed and amplified using TransPlex® Whole Transcriptome Amplification Kit (Sigma-Aldrich®, Schnelldorf, Germany) with subsequent purification using QIAquick PCR Purification Kit (QIAGEN, Germany), according to manufacturer's instructions. Expression levels of PRMT6 and BGUS, a housekeeping gene used as endogenous control, were evaluated using TaqMan® Gene Expression Assay (Applied Biosystems®, Life Technologies™, Foster City, CA, USA) (Hs_00250803_s1 and Hs_99999908_m1, respectively). To calculate the relative expression levels in each sample, the expression values of PRMT6 were normalized using the median of the reference gene (BGUS) to obtain a ratio (PRMT6/GUSB). Each plate included 2 negative controls and five sequential dilutions of a cDNA from human prostate RNA (Ambion®, Invitrogen, Carlsbad, CA, USA) to construct a standard curve for each plate. All experiments were run in triplicate.

4 IMMUNOHISTOCHEMISTRY

Histological slides from FFPE tissue fragments were achieved from the same surgical specimens used in clinical sample series through the realization of 4µm thickness histological sections. Firstly, slides were deparaffinized in xylene (Sigma-Aldrich®, St. Louis, MO, USA) and then hydrated in a decreasing series of ethanol solutions (Merck, Darmstadt, Germany). Epitope retrieval was performed with ethylenediaminetetraacetic acid (EDTA) buffer (Thermo Scientific, Waltham, MA, USA) for 20 minutes, in a microwave at 700W. Endogenous peroxidase activity was neutralized for 20 minutes with 0.6% hydrogen peroxide (Merck). Protein detection was performed using the Novolink™ Max Polymer Detection System (Leica Biosystems, Nussloch, Germany), according to manufacturer instructions. Slides were incubated overnight with a mouse monoclonal antibody specific for PRMT6 (sc-271744; Santa Cruz Biotechnology Inc., Santa Cruz, CA) in a 1:150 dilution at 4ºC, inside a humid chamber. Subsequent washing steps were performed with tris buffered saline with Tween® 20 (TBS-T) (Sigma-Aldrich®). Antigen-antibody binding reaction was revealed through the slides incubation for 7 minutes, in the dark, in a 0.05% (m/v) 3,3'-diaminobenzidine (DAB) solution (Sigma-Aldrich®) in phosphate-buffered saline (PBS) (Biochrom Ltd., Cambridge, United Kingdom) previously activated with a 0,1% hydrogen peroxide solution. Counterstaining of the slides were obtained with hematoxylin (Merck) for about 5 seconds and then slides were washed for 1 minute in a 0.25% ammonium

solution (Merck). Lastly, the slides were dehydrated in an increasing series of ethanol content and diaphanized in xylene. After the coverslip was mounted, slides were dried. For positive control of the immunohistochemistry (IHC) reaction, FFPE tissue from a normal testis was also included.

Slides were observed at the optical microscope and evaluated for PRMT6 immunoexpression by an experienced Uropathologist. Scoring criteria were adapted from a previous publication of our research group [116]: +1 for weak expression in ≤ 50% of cells; +2 for weak expression in > 50% of cells or moderate expression in ≤ 50% of cells; +3 for moderate expression in > 50% of cells or intense expression (which typically occurred in more than 50% of cells).

5 CELL CULTURE

In present study, although five PCa cell lines (LNCaP, 22RV1, VCaP, PC-3 and DU145) were firstly screened for PRMT6 expression, only LNCaP and PC-3 were maintained in culture for further *in vitro* studies. Concerning these two PCa cell lines, LNCaP is a hormone-sensitive and PC-3 is a hormone refractory cell line. Both LNCaP and PC-3 were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at The Institute for Cancer Research at Oslo, Norway.

Cell lines were grown using the recommended medium and correspondent supplements (Table 3) and 1% of Penicillin-Streptomycin (P-S) (GIBCO, Invitrogen, Carlsbad, CA, USA), in an humidifying chamber (37ºC and 5% CO_2). TrypLE™ Express (GIBCO) dissociation reagent allowed subculture the selected cell lines to harvest them. Additionally, the cultured cell lines were routinely tested by a specific multiplex PCR for contamination by *Mycoplasma spp*.

Table 3: Culture media conditions for PCa cell lines

PCA CELL LINE	GROWTH MEDIUM	SUPPLEMENTS
PC-3	RPMI-1640 + F-12	10% Fetal bovine serum (FBS)
LNCAP	RPMI-1640	

6 *PRMT6* KNOCKDOWN IN SELECTED PCa CELL LINES

PRMT6 knockdown was achieved through viral transduction in LNCaP and PC3 cell lines using MISSION® shRNA Lentiviral Transduction Particles (SHCLNV_TRCN0000299934 for LNCaP cell line and SHCLNV_TRCN0000299933 for PC-3 cell line; Sigma-Aldrich®) in the presence of polybrene (Santa Cruz Biotechnology Inc.) as described by the manufacturer. Moreover, control LNCaP and PC3 cells were generated using a MISSION® Non-Mammalian shRNA Control Transduction Particles (SHC002V; Sigma-Aldrich®). After transduction, stable clones with shRNA were selected with Puromycin dihydrochloride (cat. 631306, Clontech Laboratories Inc.) at a final concentration of 3µg/mL or 5µg/mL in LNCaP or PC3 cells, respectively.

7 RNA EXTRATION FROM SILENCED CELL LINES

Cell culture flasks (75 cm^3) at 100% confluence with LNCaP and PC-3 *PRMT6* silenced cells were harvested with a dissociation reagent, TrypLE™ Express (GIBCO®) and centrifuged for 5 minutes at 1,200 rpm. Cell pellets were ressuspended in 1 mL of PBS (GIBCO®), and centrifuged for 5 minutes at 1,200 rpm. The supernatant was discarded and cell pellets were stored at -80ºC.

Total RNA from LNCaP and PC-3 *PRMT6* silenced cells was extracted by TRIzol® Reagent (Invitrogen), according to manufacturer instructions. The pellets generated from total RNA extraction were dried and eluted in RNA storage solution (1 mM sodium citrate, pH 6.4) (Ambion®, Life Technologies™, Foster City, CA, USA). The concentration, purity and integrity of RNA samples were determined as previously described and RNA samples were stored ate -80ºC.

8 CDNA SYNTHESIS

In order to evaluate PRMT6 mRNA expression in LNCaP and PC-3 *PRMT6* silenced cells, 1000ng of cDNA was synthesized from total extracted RNA. A cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®) according manufacturer instructions. Reverse transcription was performed in a Veriti® Thermal Cycler (Applied Biosystems®) and samples were then stored at -20ºC.

9 *PRMT6* EXPRESSION ASSAY

In order to confirm the efficiency of *PRMT6* knockdown in transduced cell lines, PRMT6 mRNA levels were assessed by RT-qPCR. Briefly, cDNA products were diluted 10x in distilled water and reactions were carried out in 96-well plates using a 7500 Real-Time PCR system (Applied Biosystems®). For each well, 9 µL of cDNA product were mixed with 10 µL TaqMan® Universal PCR Master Mix (Applied Biosystems®) and 1 µL TaqMan® Gene Expression Assay, specific for PRMT6 (Hs_00250803_s1, Applied Biosystems®). Each sample was run in biological and experimental triplicate, and in every plate, two negative template controls were included.

The analysis method used to ascertain *PRMT6* knockdown in selected PCa cell lines was ΔΔCt method. LNCaP and PC-3 cells transduced with MISSION® Non-Mammalian shRNA Control Transduction Particles (Sigma-Aldrich®), from now on designated as LNCaP and PC-3 Sh-Scramble, respectively, were used to normalize results for LNCaP and PC-3 cells treated with MISSION® shRNA Lentiviral Transduction Particles specific for PRMT6, from now on designated LNCaP and PC-3 Sh-PRMT6. BGUS was used as a reference gene to normalize results obtained for the *PRMT6* gene.

10 PROTEIN EXTRACTION AND QUANTIFICATION

Protein was extracted from whole-cell lysates using the Kinexus Lysis Buffer with Lysis Buffer Cocktail (Kinexus Bioinformatics Corporation, Vancouver, British Columbia, Canada). Briefly, growth medium was removed from 75 cm^3 cell-culture flasks, and cells were washed with PBS to remove the residual culture medium. After PBS removal, 200 µL of Kinexus Lysis Buffer with Lysis Buffer Cocktail were added to each flask. Cells were scrapped with the help of a cell scrapper (Santa Cruz Biotechnology Inc.) to stimulate cell detachment and lysis, being then collected to a 1.5 mL tube. Cell lysate was sonicated in ice for 4 x 10 seconds cycles, with 10 seconds gap between each cycle. After that, tubes containing cell lysates were centrifuged for 30 minutes at maximum speed at 4ºC. Supernatant was carefully transferred to a new 1.5 mL tube.

Protein concentration was determined using BCA assay (Thermo Scientific, Waltham, MA, USA) according to manufacturer's information and extracted protein samples were stored at -80ºC.

11 WESTERN BLOT ANALYSIS

PRMT6 gene silencing and alterations in the different protein levels were assessed by Western Blot analysis. Briefly, 30 µg of total protein was heated for 5 minutes at 95ºC to promote protein denaturation, centrifuged and finally loaded in a 10% sodium dodecyl sulfate polyacrylamide gel, for further electrophoresis (SDS-PAGE). Proteins were blotted onto 0.2 µm nitrocellulose or PVDF membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA) and after their blocking membranes were incubated overnight at 4ºC with primary antibody (detailed information about primary antibodies in Table 4). The blots were developed with the Clarity™ Western ECL Substrate (Bio-Rad Laboratories Inc.) and Amersham™ Hyperfilm ECL (GE™ Healthcare, Buckinghamshire, United Kingdom), to which membranes were exposed.

To ascertain equal loading of protein, the membranes were stripped with Antibody Erasing Buffer (Komabiotech, Seoul, South Korea) and reprobed with a monoclonal mouse antibody against β-Actin (1:8000, Sigma-Aldrich®) or with a rabbit polyclonal antibody against Histone H3 (1:500, Abcam).

Table 4: Essential information about the primary antibodies used in this study.

PRIMARY ANTIBODY	DILUTION	HOST	COMPANY
ANTI-PRMT6	1:500	Rabbit pAb	Novus Biologicals (Littleton, CO, USA)
ANTI-H3R2ME2A	1:500	Rabbit pAb	Novus Biologicals
ANTI-H3K4ME3	1:500	Rabbit pAb	Abcam
ANTI-AKT	1:500	Mouse mAb	Santa Cruz Biotechnologies Inc.
ANTI-PAKT	1:500	Mouse mAb	Millipore (Billerica, MA, USA)
ANTI-MTOR	1:1000	Rabbit mAb	Cell Signaling Technology (Cell Signaling Technology, Inc., Danvers, MA, USA)
ANTI-P21	1:500	Mouse mAb	BD Biosciences (Franklin Lakes, NJ, USA)
ANTI-P27	1:500	Mouse mAb	Transduction Laboratories (BD Biosciences, Franklin Lakes, NJ, USA)
ANTI-AR	1:250	Rabbit pAb	Cell Signalling
ANTI-PSA	1:6000	Rabbit mAb	Abcam

pAb: Polyclonal antibody; mAb: monoclonal antibody

12 CELL VIABILITY ASSAY

The effect of *PRMT6* on LNCaP and PC-3 cell lines viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium (MTT; Sigma-Aldrich®) assay. This assay consists on the cleavage of yellow-colored, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide, into a blue-colored formazan by the mitochondrial enzyme succinate-dehydrogenase. Briefly, the cells were seeded in 96-well plates (Sarstedt, Numbrecht, Germany) at different concentrations (Table 5), depending on cell line, in 200µL of complete medium and incubated in a humidified chamber at 37ºC and 5% CO_2. The viability assay was performed after cells adhered to the plate (0 hours) and in subsequent days (24h, 48h and 72h). On each viability assay, cells were incubated with a solution of MTT (Sigma-Aldrich®) diluted in complete medium at 37ºC for 2h. Then, MTT solution was removed, formazan crystals were dissolved in Dimethyl sulfoxide

DMSO (Sigma-Aldrich®) and plates were shaken for 15 minutes for complete dissolution.

An automated plate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany) at 540nm with a reference filter of 630nm allowed for colorimetric quantification. The optical density (OD) was directly proportional to the number of viable cells. Three biological independent experiments were performed with methodological triplicates for each experiment.

Table 5: Number of cells plated for *in vitro* experiments in PCa cell lines used in this study.

CELL LINE	NUMBER OF CELLS PER WELL (96-WELL PLATES)
PC-3	4000
LNCAP	8000

13 APOPTOSIS ASSAY

Apoptotic levels were assessed using the APOPercentage™ kit (Biocolor Ltd., Newtownabbey, Northern Ireland, UK). Shortly, this assay consists in APOPercentage dye incorporation by cells undergoing apoptosis through phosphatidylserine transmembrane movements.

Cells were grown in 96-well plates (Sarstedt) at different concentrations depending on cell line (Table 5), in complete medium and incubated in a humidified chamber at 37ºC and 5% CO_2. 72h after cell adherence to the 96-well plate, the APOPercentage assay was performed according to manufacturer's instructions. Quantification of apoptosis was performed using a FLUOstar Omega microplate reader (BMG Labtech), by the OD measurement of the released dye at 550nm with a reference filter of 620 nm. To normalize the OD measured in the apoptotic test to the cell number, the OD of apoptosis assay was divided by the OD of the viability assay, also performed in 96-well plates. The results of the apoptosis assay on the silenced cells were expressed as the ratio of the values obtained for scramble cells (set as 100%). Three biological independent experiments were performed with methodological triplicates for each experiment.

14 MIGRATION ASSAY

The impact of *PRMT6* silencing in migration ability of PC-3 cell line into a defect in a monolayer culture was determined using the wound-healing assay. Briefly, cells were grown (37ºC and 5% CO_2 atmosphere) to full confluence in 24-well plates, the medium was removed and scratches were performed using a 100µL tip. Cells were then washed with PBS and medium replaced. Scratch closure was analyzed under the inverted microscope and images were captured at different time points. Three biological independent experiments were performed with methodological quadruplicates for each experiment.

15 INVASION ASSAY

Invasion capacity of PC-3 *PRMT6* silenced cells was evaluated through Biocoat Matrigel Invasion Chambers (BD Biosciences) according to manufacturer's protocol (Figure 13). Briefly, $2.5x10^4$ cells in 500µL of serum-free medium were seeded in Matrigel inserts. Additionally, for each well plated with Matrigel inserts, the lower portion of insert was completed with 500µL of medium with serum as a chemoattractant. Then, plates were incubated in a humidified chamber at 37ºC and 5% CO_2 for 24h. Non-invading cells were removed from the top of the insert and cells that migrated were fixed in methanol and stained with VECTASHIELD® Mounting Media containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Migrated cells were manually counted under a fluorescence microscope and results were displayed as percentages of invasion relative to scramble. Three biological independent experiments were performed with methodological duplicates for each experiment.

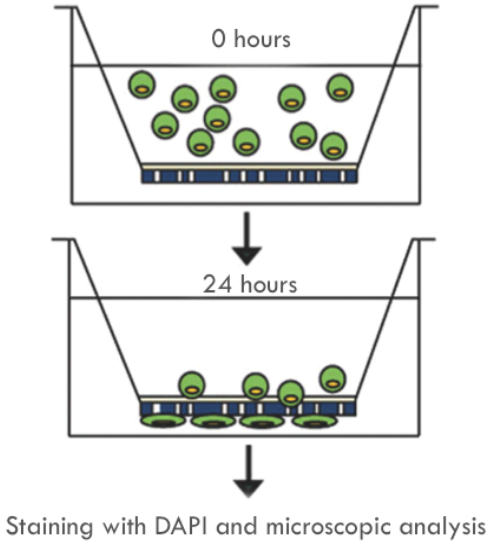
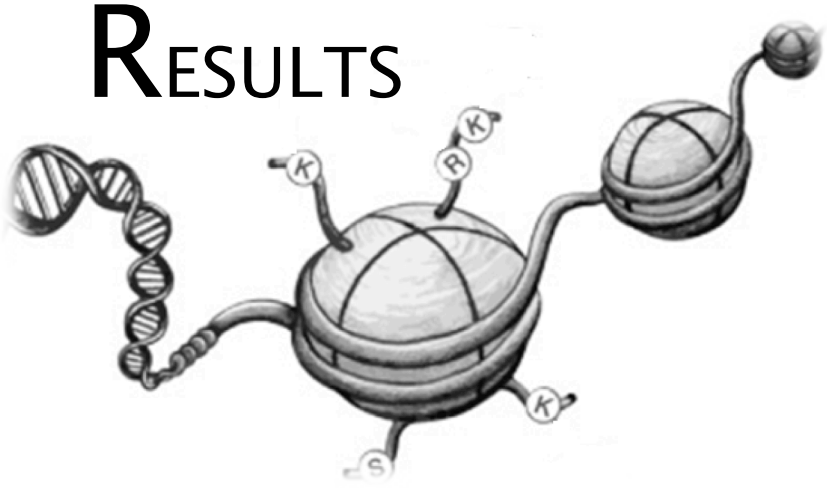


Figure 13: Principle of BioCoat Matrigel Invasion Chamber and subsequent analysis of invasion assay. (Adapted from www.bmglabtech.com)

16 STATISTICAL ANALYSIS

In order to ascertain statistical significance for comparisons made in this work, non-parametric test were used. Kruskal-Wallis test was used for comparisons between more than two non-related groups, and Mann-Whitney U test was used to compare two non-related groups. These tests were used both in clinical samples and *in vitro* studies. A receiver operator characteristic (ROC) curve was used to assess the performance of PRMT6 as diagnostic biomarker. A Spearman Correlation was used to test an association between transcript levels of different genes. Wilcoxon Signed Rank test, another non-parametric test, was used to compare related samples from the same individual in study. Fisher's Exact Test was used to measure the association between prostate tissues and immunoexpression. *P*-values were considered statistically significant when inferior to 0.05. When comparing multiple groups, Bonferroni's correction was applied in subsequent paired comparisons, dividing the P-value by the number of comparisons performed (0.017, in the case of the present study). Statistical analyses were performed using SPSS software, version 22.0 (IBM-SPSS Inc., Chicago, IL, USA). Graphs were built using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results



1 PRMT6 GENE EXPRESSION IN A LARGE SERIES OF CLINICAL SAMPLES

RT-qPCR assessment of PRMT6 mRNA levels was performed in a large series of 248 tissue samples (NPT=15; PIN=38; PCa=195). Figure 14 illustrates the overexpression of PRMT6 found in PIN lesions and in PCa samples compared to NPTs.

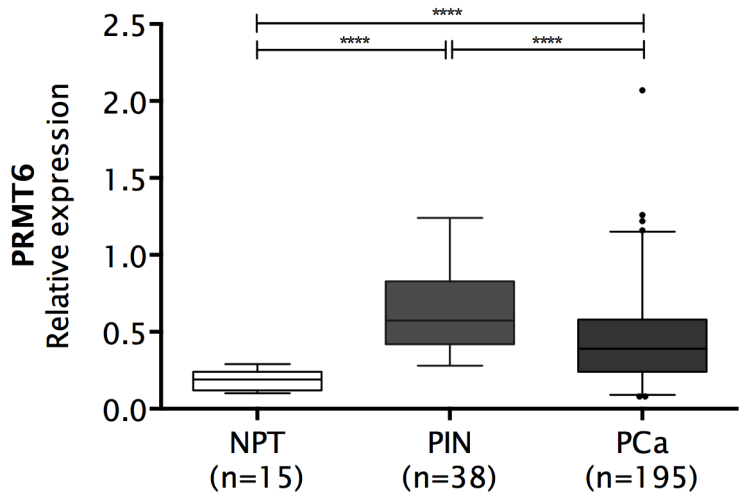


Figure 14: PRMT6 relative expression in prostate tissues. There is overexpression in PIN and PCa compared to NPTs. ****p<0.0001 (Mann-Whitney U-test).

2 ASSOCIATION BETWEEN PRMT6 EXPRESSION LEVELS AND CLINICOPATHOLOGICAL FEATURES

Relevant clinical and pathological characteristics of patients included in this study are depicted in Table 6.

Briefly, NPT, PIN and PCa groups did not significantly differ in age distribution. Additionally, no statistically significant association was found between PRMT6 expression levels and clinicopathological parameters, such as serum PSA level, GS or pathological stage (pTNM).

Table 6: Clinical and pathological data of patients included in this study

	NPT	PIN	PCA
NUMBER OF CASES, N	15	38	195
AGE (YEARS)			
Median (range)	64 (45-80)	65 (51-76)	64 (49-75)
PSA LEVELS (NG/ML)			
Median (range)	*N.A.*	*N.A.*	8.24 (0-23)
PATHOLOGICAL STAGE, N (%)			
pT2	*N.A.*	*N.A.*	109 (56%)
pT3a			67 (34%)
pT3b			19 (10%)
GLEASON SCORE, N (%)			
< 7	*N.A.*	*N.A.*	66 (34%)
= 7			115 (59%)
≥ 7			14 (7%)

N.A., Not applicable

3 EVALUATION OF PRMT6 EXPRESSION AS DIAGNOSTIC BIOMARKER

Given the PRMT6 expression patterns observed in our series, we assessed the potential performance of this enzyme as diagnostic biomarker for PCa. ROC curve analysis was performed for that purpose, enabling the definition of the best sensitivity and specificity values for an empirical cut-off expression level of 0.265. PRMT6 performance as diagnostic biomarker for PCa is depicted in Figure 15 and Table 7.

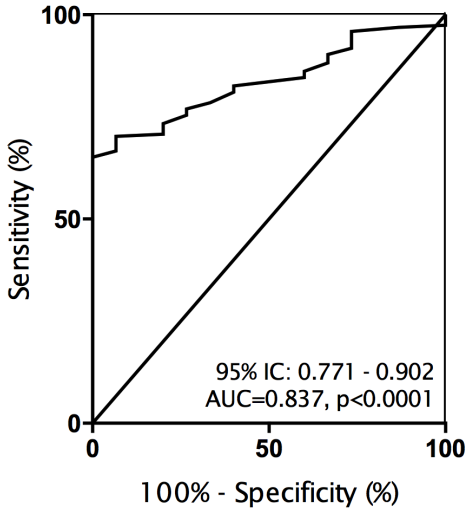


Figure 15: Performance of PRMT6 mRNA expression as a biomarker for PCa. ROC curve evaluating the ability of PRMT6 expression levels in discriminating PCa from normal prostate tissues. (AUC, area under curve; CI, confidence interval).

Table 7: Description of PRMT6 performance as diagnostic biomarker of prostate malignancy

BIOMARKER CHARACTERISTIC	VALUE
SENSITIVITY (%)	70.3 %
SPECIFICITY (%)	93.0 %
POSITIVE PREDICTIVE VALUE (PPV) (%)	99.3 %
NEGATIVE PREDICTIVE VALUE (NPV) (%)	93.0 %
ACCURACY (%)	71.9 %

4 PRMT6 EXPRESSION OF MATCHED PIN AND PCa SAMPLES

All the PIN lesions used in this study were matched with a primary PCa sample, deriving from the same patient. A Wilcoxon Signed Rank test was then performed to assess differences in PRMT6 expression levels between matched PIN and PCa. As depicted in Figure 16, expression levels were significantly higher in PIN lesions, compared to the respective PCa ($p < 0.001$).

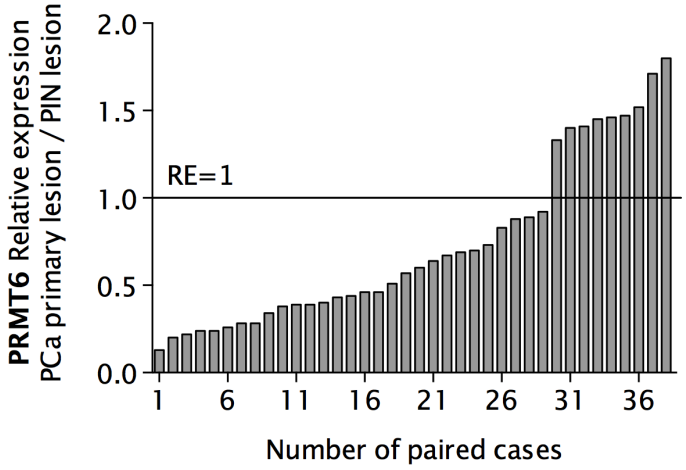


Figure 16: Relative expression of PRMT6 in paired PIN and PCa tissue samples.

5 PRMT6 PROTEIN LEVELS IN A LARGE SERIES OF CLINICAL SAMPLES

To assess PRMT6 protein expression in tissue samples, IHC was performed in a series of 248 FFPE prostate tissue samples (NPT=15; PIN=38; PCa=195) that perfectly matched with series of frozen tissues utilized for assessment of PRMT6 mRNA expression. Table 8 depicts the distribution of IHC scores in each group of samples and illustrative examples are presented in Figure 17.

Table 8: Immunohistochemical expression of PRMT6 in a series of NPT, PIN lesions and primary PCa.

CLINICAL SAMPLE GROUP	SCORE +1 N (%)	SCORE +2 N (%)	SCORE +3 N (%)
NPT	15 (100%)	-	-
PIN	14 (37%)	15 (39%)	9 (24%)
PCA	100 (51%)	63 (32%)	32 (16%)

A significant increase of PRMT6 protein levels from NPTs to PIN lesions and PCa samples is apparent. Score distribution of PRMT6 immunostaining across the three groups of clinical samples is illustrated in Figure 18 - A. Paralleling PRMT6 mRNA expression results, the highest percentage of cases scored as +2 and +3 were found in PIN.

A statistically significant association between *PRMT6* transcript and protein levels was apparent ($p < 0.001$), indicating that, globally, PRMT6 protein levels follow the same trend as that of the transcript (Figure 18 – B). In pairwise comparisons, statistical significance was detected between scores +1 *vs* +2 and +1 *vs* +3 ($p < 0.01$ and $p < 0.001$, respectively). Additionally, a significant association was obtained between immunoexpression and prostate tissues.

Table 9: Association between PRMT6 expression and prostate tissue.

CLINICAL SAMPLE GROUP	NORMAL EXPRESSION	OVEREXPRESSION	P
NPT	15 (100%)	-	****
NEOPLASTIC PROSTATE TISSUE	114 (49%)	119 (51%)	

Neoplastic Prostate Tissue: PIN lesions and PCa samples; Normal Expression: +1; Overexpression: +2 and +3; ****p<0.0001 (Fisher´s Exact Test).

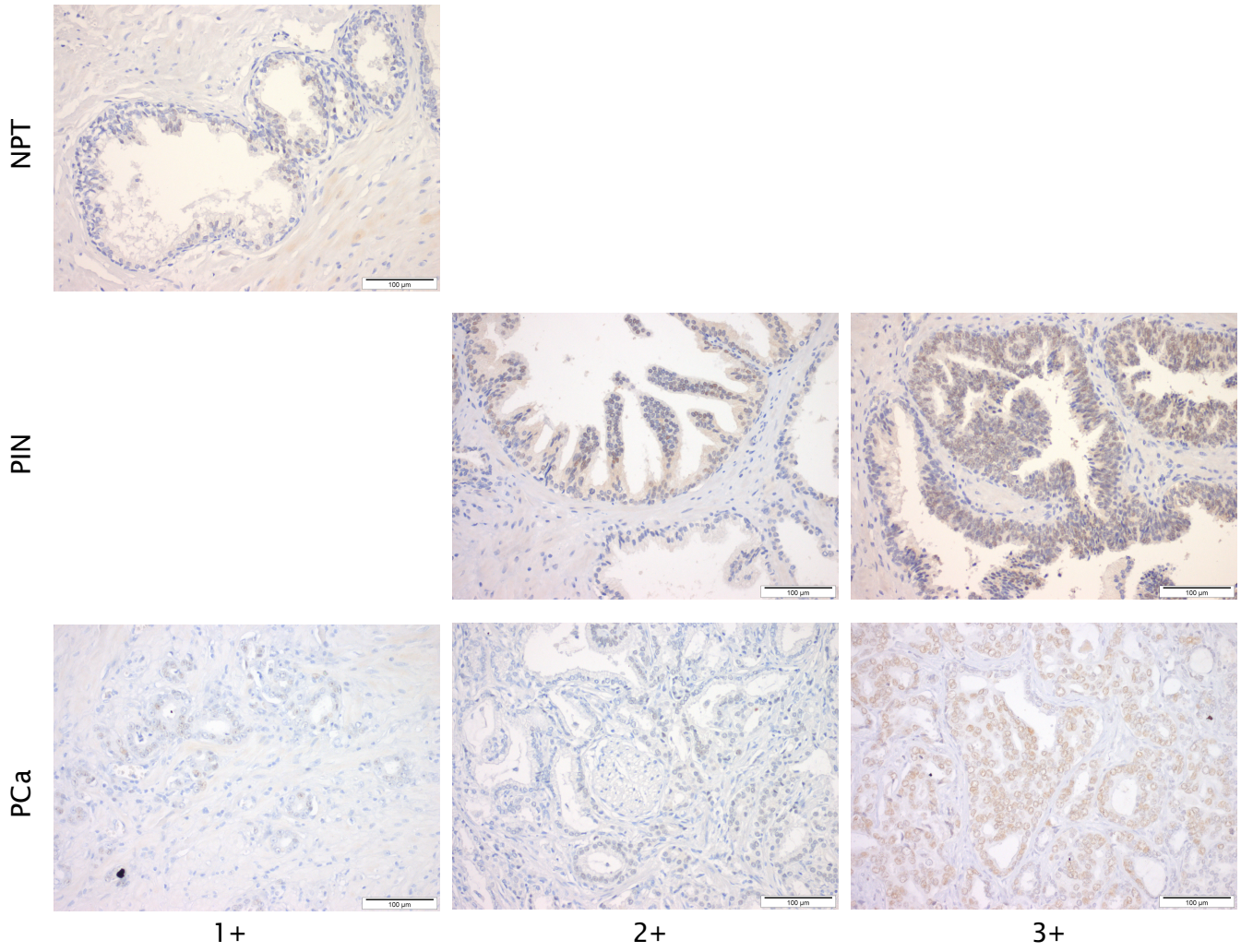


Figure 17: Illustrative images of PRMT6 immunostaining in NPT, PIN and PCa samples.

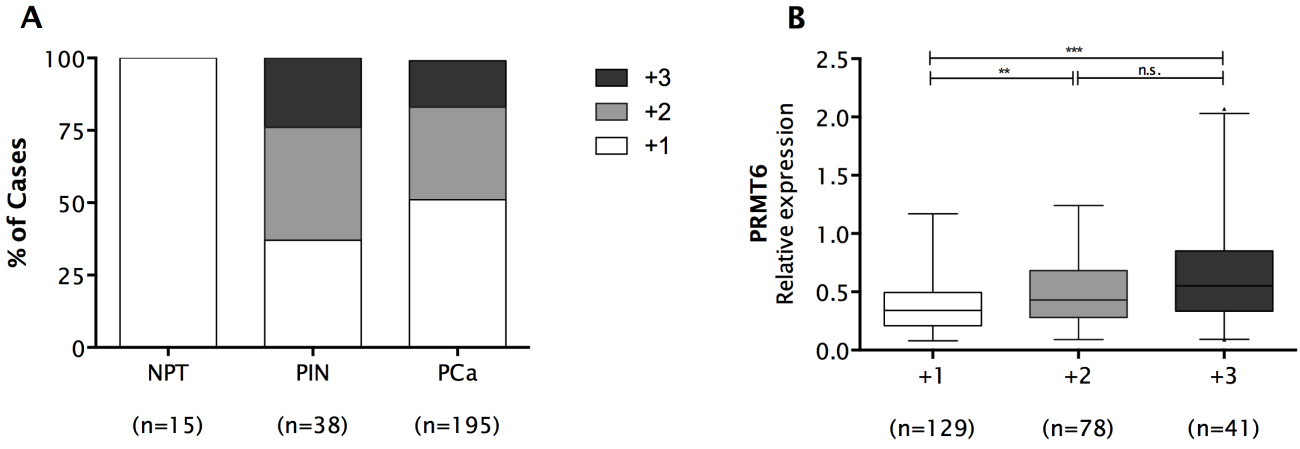


Figure 18: Distribution of PRMT6 immunoexpression (A) and of PRMT6 transcript levels (B) in a series of prostate tissue samples (NPT, PIN and PCa), grouped according to PRMT6 immunostaining. **p<0.01;***p<0.001;n.s. non significant (Mann-Whitney U-test).

6 KNOCKDOWN OF PRMT6 IN PC-3 AND LNCaP CELL LINES

Firstly, PRMT6 expression levels were assessed by RT-qPCR in five PCa cell lines (LNCaP, 22RV1, VCaP, PC-3 and DU145) (Figure 19-A). All cell lines expressed PRMT6, although at variable levels. Then, the androgen responsive and the androgen refractory cell lines that expressed higher levels of PRMT6 (LNCaP and PC-3, respectively) were chosen for *in vitro* studies. Furthermore, effective PRMT6 knockdown was achieved in the selected cell lines and confirmed both at mRNA and protein level (Figure 19-B).

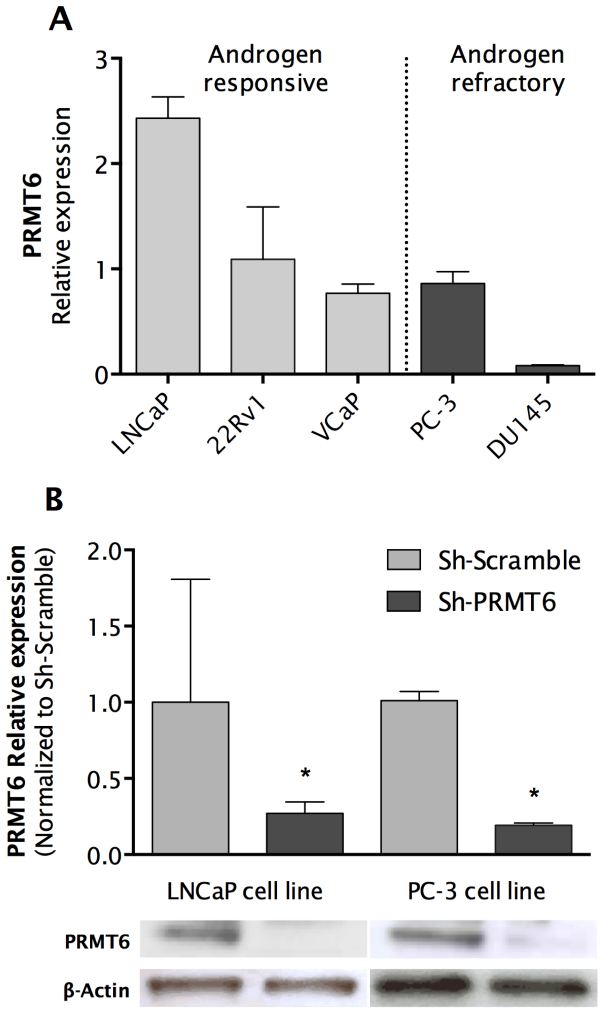


Figure 19: (A) PRMT6 expression in PCa cell lines. (B) The efficiency of PRMT6 knockdown was confirmed at mRNA, using RT-qPCR (upper panel), and protein level, using Western-Blot (lower panel), in LNCaP and PC-3 cells. *p<0.05 (Mann-Whitney U-test).

7 IMPACT ON VIABILITY AND APOPTOSIS OF PRMT6 KNOCKDOWN IN PC-3 AND LNCaP CELL LINES

In LNCaP PRMT6-knocked down cell line, the MTT assay showed a 33% and 31% increase in cell viability at 24h and 48h, respectively. However, at 72h cell viability was similar to that of Sh-scramble (Figure 20-A). Concerning PC-3, a significant decrease in cell viability was observed after PRMT6 knockdown, both at 48h and 72h (10% and 24%, respectively) (Figure 20-B). Moreover, knockdown of PRMT6 led to an increase of apoptosis, in both LNCaP and PC-3 cell lines. Nevertheless, this increase was only statistically significant in PC-3 cells (Figure 20-C and D).

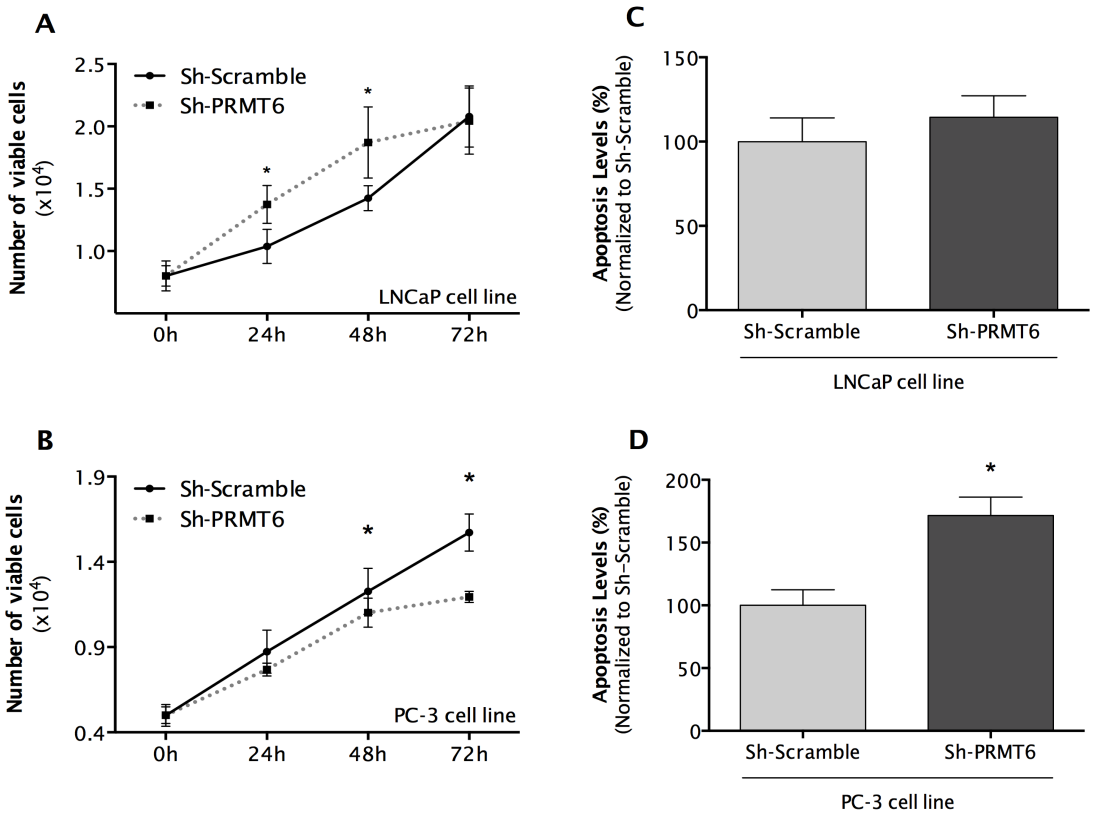


Figure 20: Impact of PRMT6 knockdown in cell viability and apoptosis of LNCaP and PC-3 cell lines. (A) Cell viability in LNCaP and (B) cell viability in PC-3: quantification of cell viability by MTT assay in Sh-Scramble and Sh-PRMT6 at 0h, 24h, 48h and 72h. Quantification of apoptosis by APOPercentage in Sh-Scramble, Sh-PRMT6 LNCaP (C) and PC-3 (D) at 72h. *p<0.05 (Mann-Whitney U-test).

8 EFFECT OF PRMT6 KNOCKDOWN IN MIGRATION AND INVASION CAPACITY OF PC-3 CELLS

Because PC-3 is widely recognized as a highly invasive PCa cell line, we evaluated the impact of PRMT6 knockdown in migration and invasion potential in this cell line. A significant decrease in migration ability was observed in PC-3 PRMT6-silenced cells (Figure 21-A). Furthermore, a decrease in invasion capacity of PC-3 PRMT6-silenced cells was also observed, although it did not reach statistical significance (Figure 21-B).

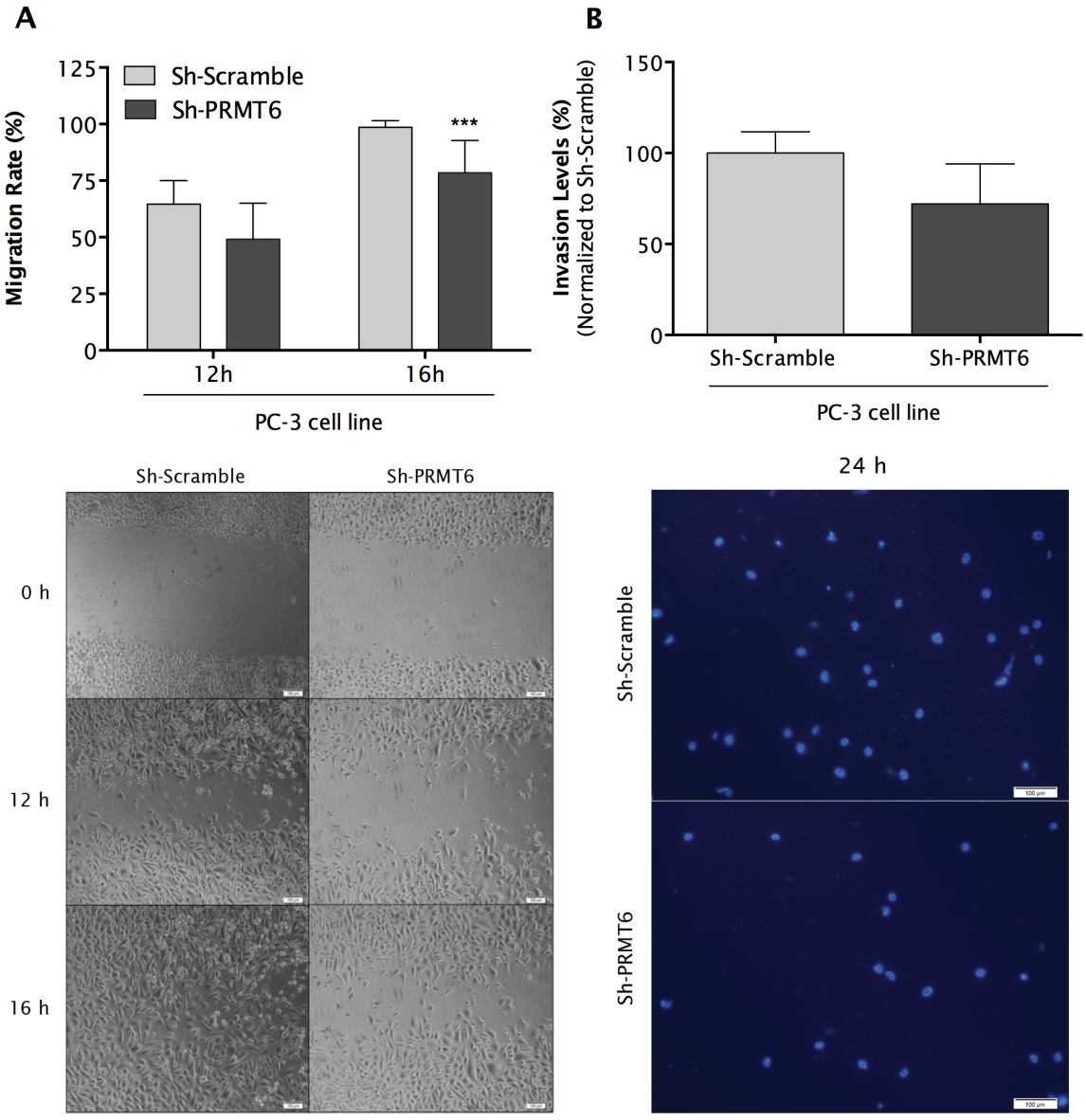


Figure 21: Impact of PRMT6 knockdown in migration and invasion properties of PC-3. (A) Wound-healing scratch assay in PC-3 cell line. The upper panel shows the migration rate at 12h and 16h, and the lower panel displays the illustrative images at the beginning and endpoint of the assay. (B) Matrigel Invasion assay in PC-3. The upper panel shows the percentage of invasive cells at 24h and the lower panel displays illustrative images at endpoint of the assay (24h). ***p<0.001 (Mann-Whitney U-test).

9 EFFECT OF PRMT6 KNOCKDOWN IN HISTONE PTMs PATTERNS IN PC-3 CELL LINE

As PRMT6 knockdown produced phenotypic alterations in silenced PC-3 cells, we ascertained alterations in histone marks established by this arginine methyltransferase. Therefore, western blot was performed to quantitate H3R2me2a. The observed reduction of this mark, established by PRMT6 [117], is illustrated in Figure 22.

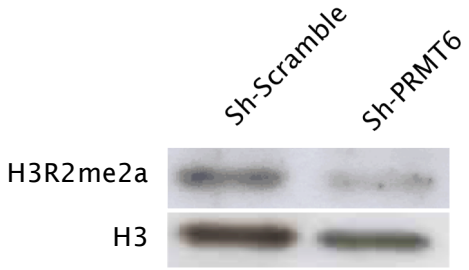


Figure 22: Reduction of asymmetrical dimethylation of arginine 2 of histone 3 after PRMT6 knockdown in PC-3 cell line.

PRMT6 methyltransferase activity in arginine 2 of histone 3 counteracts the MLL complex activity on lysine 4 of the same histone, being mutually exclusive [117-119]. As PRMT6 was overexpressed in PCa samples, we evaluated the impact of this deregulation in MLL complex expression and activity. We had previously found that MLL family genes (MLL1-5) were downregulated in PCa [120]. Because the case series used in the present study is partially common to that of our previous publication, we interrogated the combined datasets concerning a putative association between PRMT6 and MLL complex expression. Although Spearman correlation analysis did not disclose an association between PRMT6 and MLL complex transcript levels in primary PCa tissues, PRMT6 knockdown was associated with MLL3-5 upregulation in PC-3 cells (Figure 23). The same trend was observed for SMYD3, another H3K4me3 methyltransferase (Figure 23).

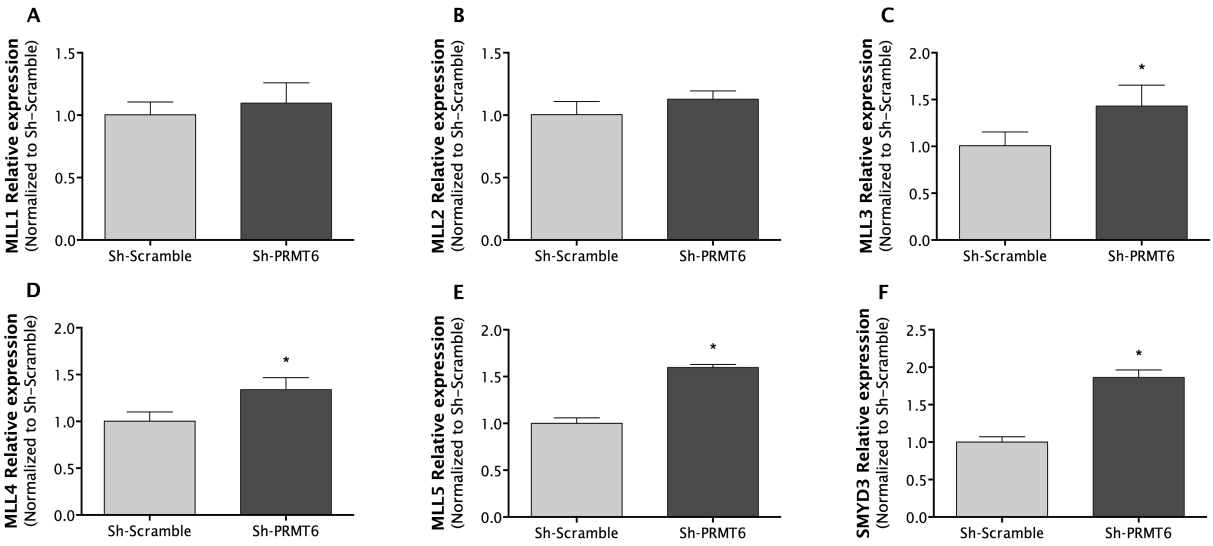


Figure 23: Relative expression of methyltransferases that catalyze H3K4me3 in PC3 PRMT6-silenced cells. (A-E) MLL complex genes are upregulated in PC3 Sh-PRMT6 cells, reaching statistical significance for MLL3-5. (F) SMYD3 is also significantly upregulated in PC3 knocked down cells. *p<0.05 (Mann-Whitney U-test).

Moreover, the impact of PRMT6 knockdown on MLL complex activity *in vitro* was tested through the evaluation of the differences in H3K4me3 expression, in PC-3 cells. However, no significant alterations in expression were apparent after PRMT6 knockdown (Figure 24 – A).

Because PRMT6 activity is associated with a subset of transcriptionally inactive Hox genes and presumably also with some MYC-dependent genes [117], we evaluated MYC transcript levels before and after PRMT6 silencing. RT-qPCR analysis depicted a significant increase in MYC mRNA levels in PC-3 Sh-PRMT6 (Figure 24 - B).

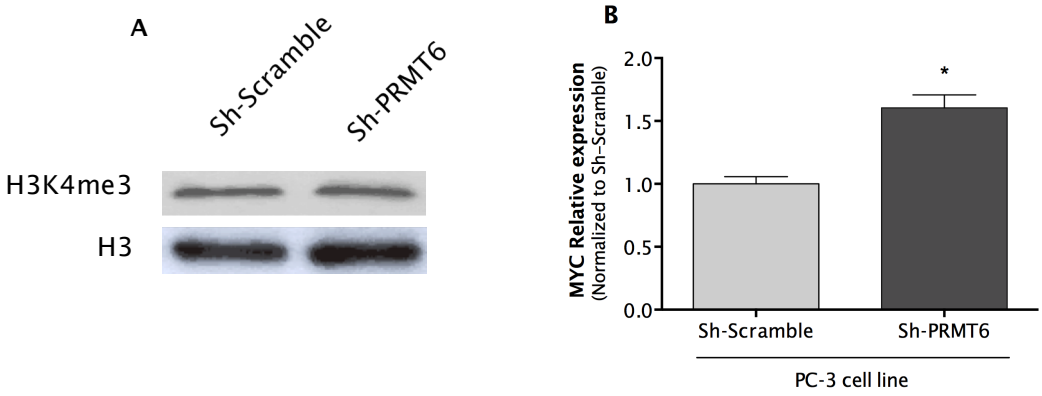


Figure 24: H3K4me3 and MYC expression after PRMT6 knockdown in PC-3 cell line. (A) Western blot analysis of H3K4me3 expression after PRMT6 knockdown in PC-3 cells. (B) MYC transcript levels in PC-3 PRMT6 silenced cells. *p<0.05 (Mann-Whitney U-test).

10 MOLECULAR PATHWAYS AND PUTATIVE TARGET GENES REGULATED BY PRMT6

To find genes regulated by PRMT6, we evaluated putative targets associated with cellular processes and pathways relevant in PCa. Firstly, cellular senescence was investigated, since this biological process has been previously associated with PRMT6 activity [121, 122].

Among molecules responsible for induction of cellular senescence, p21 and p27 play a major role [121-123]. In PC-3 Sh-PRMT6 cells, western blot analysis showed a considerable increase of p27 expression whilst p21 was only slightly increased (Figure 25).

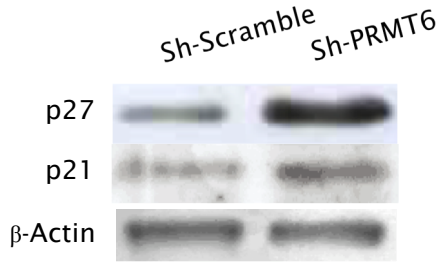


Figure 25: Western blot analysis of p21 and p27 expression in PC-3 cell line after PRMT6 knockdown.

Considering the decrease in invasion and migration ability of PC-3 Sh-PRMT6 cells, we further evaluated the expression levels of genes involved in those cellular processes. We found that PRMT6 silencing was associated with matrix metalloproteinase 9 (MMP-9) downregulation and CD44 upregulation (Figure 26).

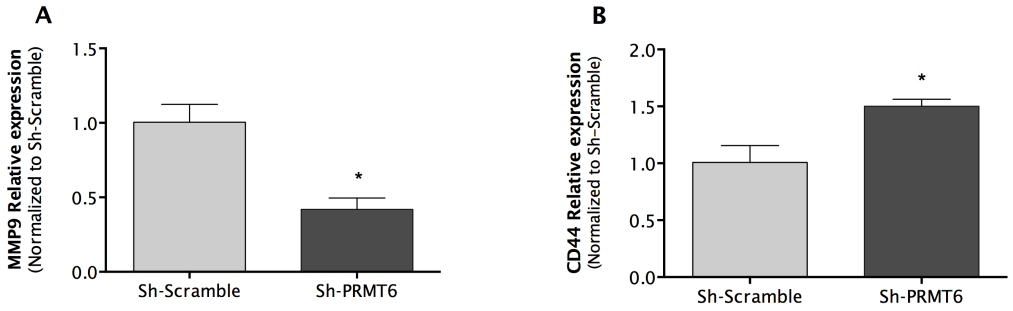


Figure 26: Impact of PRMT6 knockdown in MMP-9 and CD44 transcript levels in PC-3 cell line. *p<0,05 (Mann-Whitney U-test).

The PI3K/AKT/mTOR pathway, which is frequently deregulated in PCa was also investigated [124]. In PC-3 Sh-PRMT6 cells, AKT, phosphorylated AKT and mTOR protein expression was significantly decreased (Figure 27), suggesting PI3K/AKT/mTOR pathway downregulation due to PRMT6 silencing.

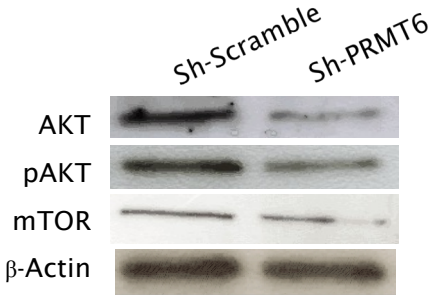


Figure 27: Western blot analysis of AKT, pAKT and mTOR expression in PC-3 cell line.

Finally, the AR pathway, which is critically involved in PCa initiation and progression [125], was examined. In PC-3 cells, which have only residual expression of AR [126], PRMT6 knockdown led to AR upregulation, both at transcript and protein level (Figure 28). To confirm these results, expression of PSA (a well-known downstream target of AR) was assessed, and an impressive increase in PSA protein levels was apparent in PC-3 Sh-PRMT6 cells (Figure 28 - B).

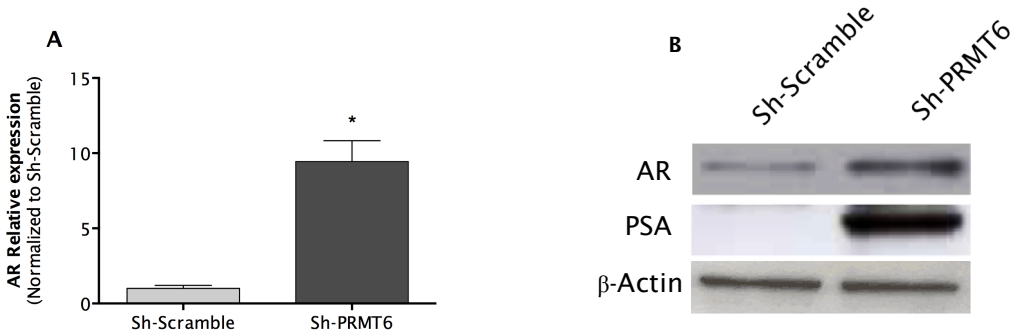
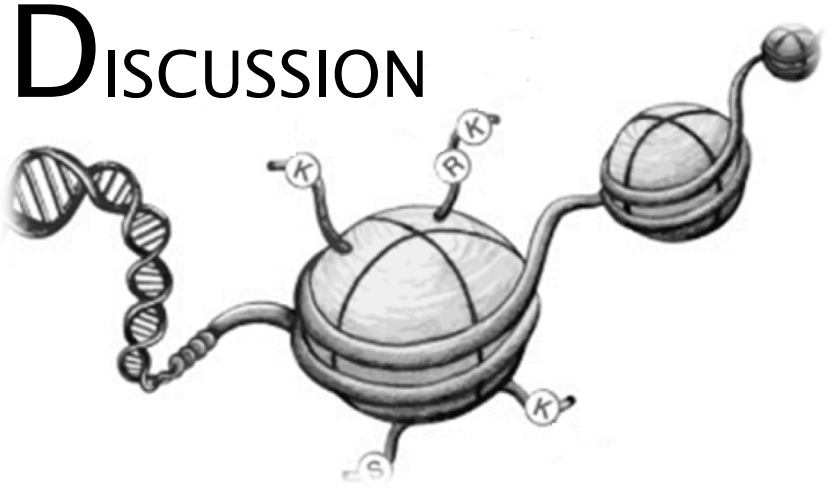


Figure 23: Overexpression of AR in PC-3 PRMT6 silenced cells. RT-qPCR analysis of AR transcript levels in PC-3 cell line (left panel). Western blot analysis of AR and PSA protein levels in PC-3 cell line. *p<0,05 (Mann-Whitney U-test).

Discussion



1 DISCUSSION

PCa is one of the most prevalent human malignancies, constituting a leading cause of morbidity and mortality. Over the last decades, PCa epidemiology has changed significantly. The widespread use of serum PSA testing as a PCa biomarker resulted in an anticipation of the median age of diagnosis and downstaging. Indeed, from a cancer mostly diagnosed in men over 70 years-old and with clinically advanced disease, many PCa cases are now diagnosed in asymptomatic men aged 50 to 70 years, mainly with clinically localized disease and, therefore, with a greater chance of cure. However, the downside of this trend has been the increasing awareness of overdiagnosis and subsequent overtreatment due to the fact that serum PSA testing is unable to identify life-threatening PCa. Because currently used clinical and pathological tools do not accurately distinguish clinically indolent from aggressive tumors, a better understanding of prostatic carcinogenesis is required to improve diagnosis and management of this malignancy.

Deregulation of epigenetic mechanisms has been increasingly recognized as a key player in tumorigenesis [57]. However, only more recently the critical role of altered patterns of histone post-translational modifications and of histone-modifying enzymes activity has been emphasized [92]. Those alterations have been reported in several common cancers, including those of the prostate [92, 93]. Histone methylation has received particular attention since alterations in expression of HMTs and HDMs, as well as corresponding histone modification patterns, seems to provide useful biomarkers for diagnosis and prognostication [99-102]. Recently, our research team has shown that several HMTs and HDMs were consistently deregulated in PCa and might be of prognostic significance [120]. In that study, PRMT6 was found overexpressed in PCa tissues, eventually discriminating normal from tumorous prostate tissues [120]. Herein, we extended those preliminary finding to a larger series of PCa cases and attempted to unravel the biological impact of PRMT6 deregulation in PCa cells.

The PRMT6 argine methyltransferase, encoded by PRMT6 gene mapped at 1p13.3, is preferentially localized in the nucleus [127]. This member of type I arginine methyltransferases family displays a high affinity for R2 of H3 [117, 118]. Only PRMT6 has been proved to catalyze H3R2 asymmetric di-methylation, although PRMT4 has been documented to exert the same function, but only *in*

vitro [117]. Because H3R2me2 is a repressive mark, PRMT6 activity has strongly associated with transcriptional silencing [117]. Besides PCa, PRMT6 overexpression of this enzyme has been reported in bladder and lung cancer [98, 115]. The precise functions of PRMT6 are not completely understood at present, although it has been associated with altered cell proliferation, cellular senescence, DNA repair and innate immunity [121-123, 128, 129].

Following our previous observation of PRMT6 overexpression in PCa [120], we validated this finding in a an extended series of cases, incorporating also PIN lesions, a putative PCa precursor. We confirmed that PRMT6 is frequently overexpressed in PCa and also in PIN lesions, compared to normal prostate tissues. Unexpectadly, PIN lesions displayed significantly higher PRMT6 transcript levels than PCa, a finding that was also apparent when only matched PCa and PIN lesions were analyzed. Theoretically, owing to the precursor role of PIN, intermediate levels of PRMT6 expression would be expected. The most likely explanation is that PRMT6 oncogenic role is more relevant during the earliest phases of tumorigenesis, although it remains important during cancer progression. This hypothesis is further supported by the lack of association between PRMT6 transcript levels and clinicopathological parameters of aggressive disease. Concerning the matched analysis, it should be recalled that a direct relation between paired PIN and PCa cases can not be inferred as a biological connection was not investigated in this series.

Importantly, the variations of PRMT6 mRNA expression were confirmed at protein level, using IHC. Indeed, not only marked differences in IHC score were depicted between NPT, on the one hand, and PIN and PCa, on the other, but also the proportion of cases with +3 IHC score was higher in PIN. Interestingly, IHC scores globally correlated with PRMT6 transcript levels, although no significant differences were depicted between scores +2 and +3. This observation is probably due to the semi-quantitative nature of IHC scoring, which compressed PRMT6 expression variations into three categories, whereas the quantitation of mRNA expression levels is more discriminative. Indeed, using a cutoff value derived from ROC curve analysis to maximize performance, PRMT6 transcript levels were shown to accurately discriminate PCa from non-cancerous prostate tissues, confirming our previous findings [120] and suggesting a relevant role for PRMT6 mRNA expression as a PCa biomarker. Contrarily, PRMT6

immunoexpression is not likely to provide relevant information for PCa diagnosis as 51% of PCa samples display the same score as normal prostate tissues (+1).

To ascertain the biological role of PRMT6 in PCa, we assessed the phenotypic effect of PRMT6 knockdown in LNCaP and PC-3 cell lines, which were chosen because it displayed the highest expression levels among, respectively, the androgen-sensitive and androgen-insensitive PCa cell lines tested. After achieving stable reduction of *PRMT6* expression, cell viability, apoptosis, migration and invasion assays were carried out. Whereas an attenuation of the malignant phenotype (decrease of cell viability and increase of apoptosis) was consistently observed in PRMT6-silenced PC-3 cells, no permanent effect was apparent in PRMT6-silenced LNCaP cells. Following an initial increase in cell viability, no differences were depicted at 72h. This paradoxical behavior of LNCaP cells might be related with the AR regulating role of PRMT6. As PRMT6 activity leads to downregulation of AR, its silencing is likely to cause de-repression of AR transcription, with a positive impact in neoplastic cell viability. Thus, PC-3 was chosen for additional functional assays, testing migration and invasion capacities, which confirmed the effects of PRMT6 silencing in the attenuation of the malignant phenotype.

Owing to the specificity of PRMT6 to asymmetrically dimethylate H3R2 [117, 118], we hypothesized that *PRMT6* knockdown could lead to a reduction of global levels of that methylation mark. Thus, the observed reduction in H3R2me2a is most likely due to the specificity of PRMT6 to catalyze that mark. PRMT6 activity counteracts that of the MLL complex and, thus, we also evaluated MLL complex expression and activity after PRMT6 knockdown. As expected, an increase in MLL complex was observed, but no significant difference in H3K4me3 (the histone mark catalyzed by MLL) levels was apparent, even with concurrent SMYD3 (another methyltransferase that catalyzes H3K4me3) increased expression. Because several other enzymes (which we did not assess) catalyze the H3K4me3 mark, it is plausible to assume that variations in MLL and SMYD3 might not impact in H3K4me3 global levels. However, a gene-specific effect of those variations cannot be dismissed.

To better understand the biological role of PRMT6 in PCa at molecular levels, we selected key genes involved in cellular pathways deregulated during prostate carcinogenesis. Owing to the reported involvement of PRMT6 in cellular

senescence [122, 123, 130], p21 and p27 expression levels were assessed in Sh-scramble and Sh-PRMT6 PC-3 cells. Although both proteins were increased in Sh-PRMT6 PC-3 cells, p27 showed the most impressive variation and these results are in line with those observed in other tumor models [122, 123, 130], implying that, indeed, PRMT6 overexpression in PCa is likely to interfere with normal cellular senescence, facilitating neoplastic transformation. Remarkably, p27 seems to play an important role in prostate carcinogenesis as its expression decreases with increased tumor grade and stage [131-133] and p27 downregulation has been associated with poor prognosis [131-134]. Furthermore, a decrease in cell proliferation accompanied by an increase in cell death due to apoptosis upon induction of p27 expression has been reported in PC-3 cell line [130], a finding that corroborates our results of phenotypic assays in Sh-PRMT6 PC-3 cells.

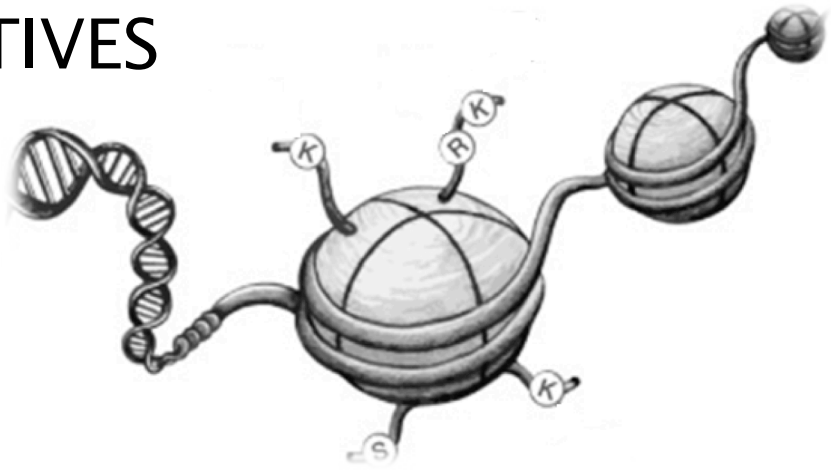
To further validate the results of the phenotypic assays, the expression of two molecules involved in tumor cell migration and invasion – MMP-9 and CD44 – was evaluated. In Sh-PRMT6 PC-3 cells, MMP-9 was found to be downregulated, whereas CD44 was upregulated. MMP-9 belongs to the group of gelatinases [135] and its role in promoting cancer invasion and metastasis is well documented in several cancer types [136-138]. Regarding CD44, a transmembrane glycoprotein involved in cell-cell and cell-matrix adhesion as well as in cell signaling, its role in cell migration, signaling and tumor metastasis is also well documented [139-141]. In PCa, decreased expression of CD44 has been reported in primary tumors, with a pronounced lack of expression in metastasis [142, 143]. Moreover, CD44 downregulation has been associated with increased grade and pathological stage in PCa, predicting tumor recurrence and biochemical failure [144-146]. Thus, the observed variations in MMP-9 and CD44 expression in association with PRMT6 expression are in line with the reported role and altered expression of these molecules in primary PCa. Interestingly, CD44 decreased expression in PCa has been associated with aberrant promoter methylation [147]. Considering our results, we might speculate whether H3R2me2a catalyzed by PRMT6 might be an additional mechanism for CD44 silencing in PCa.

Because alterations in the PI3K/AKT/mTOR pathway have been reported in 42% of primary PCa and in 100% of metastatic tumors [148], key genes of this pathway have been also investigated. In line with the predicted effect of PRMT6 silencing, downregulation of AKT, phosphor-AKT and mTOR were observed.

Although loss of expression of phosphatases and tensin homolog (PTEN), a negative regulator of PI3K/AKT/mTOR pathway, in PCa is a common explanation for the increased activity of that signaling pathway [149-151], PRMT6 activity might further provide conditions for PI3K/AKT/mTOR pathway activation. Importantly, both PTEN loss and AKT activation have been described to be associated with poor clinical outcome [152], biochemical recurrence after RP [153, 154], as well as resistance to radiation [155] and chemotherapy [156, 157].

AR signaling is critical for the normal development, function and homeostasis of the prostate gland and its deregulation has been implicated in prostate carcinogenesis and tumor progression [158]. Although primary PCa is mostly an androgen-responsive tumor, the castration-resistant phenotype eventually emerges and is associated with clinical aggressiveness and refractoriness to ADT [158]. Several mechanisms have been implicated in the emergence of CRPC, including heterogeneous loss of AR expression, associated aberrant promoter methylation in 18% to 28% of cases [159, 160]. Additionally, the lack of AR expression has been associated with increased invasiveness [161, 162]. PC-3 cells express AR at very low levels and might be, thus, considered a good model of CRPC. We found that PRMT6 silencing was associated with restored AR expression, which was functional, as demonstrated by increased PSA expression. Moreover, AR upregulation might also contribute to decreased migration and invasion capabilities documented in Sh-PRMT6 PC-3 cells. Interestingly, strategies that restore AR expression and, thus, overcome castration-resistance, might be of clinical utility [161, 162]. Hence, PRMT6 downregulation and/or inhibition might provide an innovative therapeutic strategy for CRPC, through restoration of AR expression, re-sensitizing neoplastic cells to ADT. Further studies, combining PRMT6 downregulation and exposure to anti-androgens (e.g., bicalutamide) are mandatory to ascertain the clinical viability of this therapeutic approach.

Conclusion and Future Perspectives



1 CONCLUSION AND FUTURE PERSPECTIVES

In this study we confirmed, in a large series of prostate tissue samples, that PRMT6 was overexpressed in PCa, at both transcript and protein level. Intriguingly, PIN lesions displayed significantly higher PRMT6 expression levels, compared to PCa. Furthermore, PRMT6 mRNA levels are able to discriminate cancerous from non-cancerous prostate tissues.

In stable PRMT6 knockdown models, attenuation of the malignant phenotype was consistently demonstrated in PC-3 but not in LNCaP cells, probably due to its different AR expression status. At molecular level, PRMT6 silencing was associated with decreased H3R2me2a levels and increased MLL complex and SMYD3 expression, although global H3K4me3 levels remained unchanged. Moreover, the expression of several key genes, involved in critical cellular pathways, was shown to be affected by PRMT6 downregulation, including p21, p27, MMP-9, CD44, AKT, phospho-AKT, mTOR and AR, globally supporting an oncogenic role for PRMT6.

Finally, restoration of AR expression in Sh-PRMT6 PC-3 cells, might be of clinical relevance as it may re-sensitize androgen-insensitive neoplastic cells to ADT.

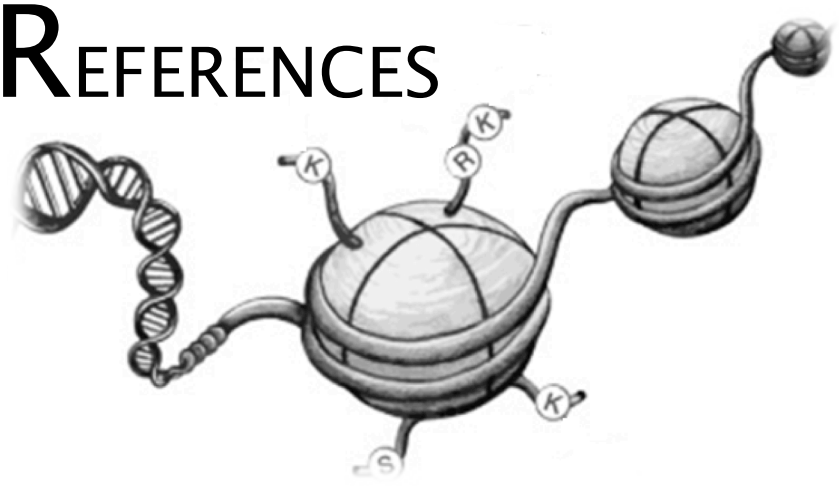
The therapeutical potential of PRMT6 silencing/inhibition is probably the most exciting finding of this study and it is surely the one that more deserves further studies. Chromatin immunoprecipitation (ChIP) experiments are required to firmly establish a link between PRMT6 activity and AR expression (de-)regulation. Furthermore, the androgen sensitivity of Sh-PRMT6 PC-3 cells must be proven by exposure to di-hydro-testosterone, as well as through combined treatment with bicalutamide, to demonstrate the re-acquisition of sensitivity to ADT.

Although results observed for Sh-PRMT6 LNCaP cells seem discouraging, the hypothesis that the paradoxical effect of PRMT6 downregulation in cell viability is due to increased AR expression must be tested. As LNCaP cells might be considered representative of androgen-sensitive PCa, which are those that predominate at diagnosis, the effects of PRMT6 inhibition/downregulation must be carefully investigated, to ascertain whether the combined therapeutic strategy

previously hypothesized for CRPC (PRMT6 inhibition and bicalutamide) might constitute a potentially harmful therapy for androgen-sensitive PCa.

Importantly, in case the combined therapy proves useful in *in vitro* models, our study provides evidence that PRMT6 expression, assessed by IHC (a commonly used technique in most Pathology labs) might serve as a predictive marker of response, through the identification of PCa cases overexpressing PRMT6, which are those that are most likely to respond.

References



1 REFERENCES

1. Oesterling JE. The origin and development of benign prostatic hyperplasia. An age-dependent process. Journal of andrology. 1991;12(6):348-55.

2. Rosai J. Rosai and Ackerman's Surgical Pathology. 10 ed: Elsevier Health Sciences; 2011.

3. Shen MM, Abate-Shen C. Molecular genetics of prostate cancer: new prospects for old challenges. Genes & development. 2010;24(18):1967-2000.

4. DeVita VT, Lawrence TS, Rosenberg SA. Cancer: Principles and Practice of Oncology-Advances in Oncology: Lippincott Williams & Wilkins; 2010.

5. Kai H. Hammerich GEA, Wheeler TM, Hammerich KH, Gustavo E. Ayala,, Wheeler. TM. Anatomy of the prostate gland and surgical pathology of prostate cancer

Prostate Cancer: Cambridge University Press; 2008.

6. Hammerich KH, Ayala GE, Wheeler TM. Anatomy of the prostate gland and surgical pathology of prostate cancer. Cambridge University, Cambridge. 2009:1-10.

7. McNeal JE. The zonal anatomy of the prostate. The Prostate. 1981;2(1):35-49.

8. Wadhera P. An introduction to acinar pressures in BPH and prostate cancer. Nature reviews Urology. 2013;10(6):358-66.

9. Papatsoris A, Kostopoulos C, Migdalis V, Chrisofos M. Prostate Cancer Precursor Diseases. 2009.

10. Alcaraz A, Hammerer P, Tubaro A, Schröder FH, Castro R. Is There Evidence of a Relationship between Benign Prostatic Hyperplasia and Prostate Cancer? Findings of a Literature Review. European urology. 2009;55(4):864-75.

11. Orsted DD, Bojesen SE. The link between benign prostatic hyperplasia and prostate cancer. Nature reviews Urology. 2013;10(1):49-54.

12. Bostwick DG, Brawer MK. Prostatic intra‐epithelial neoplasia and early invasion in prostate cancer. Cancer. 1987;59(4):788-94.

13. Bostwick DG, Cheng L. Precursors of prostate cancer. Histopathology. 2012;60(1):4-27.

14. Brawer MK. Prostatic intraepithelial neoplasia: an overview. Reviews in urology. 2005;7 Suppl 3:S11-8.

15. Bostwick DG. Prostatic adenocarcinoma following androgen deprivation therapy: the new difficulty in histologic interpretation. Anat Pathol. 1998;3:1-16.

16. De Marzo AM, Marchi VL, Epstein JI, Nelson WG. Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis. The American journal of pathology. 1999;155(6):1985-92.

17. Boyd LK, Mao X, Lu YJ. The complexity of prostate cancer: genomic alterations and heterogeneity. Nature reviews Urology. 2012;9(11):652-64.

18. Chrisofos M, Papatsoris AG, Lazaris A, Deliveliotis C. Precursor lesions of prostate cancer. Critical reviews in clinical laboratory sciences. 2007;44(3):243-70.

19. Ferlay J SI, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on day/month/year.

20. Center MM, Jemal A, Lortet-Tieulent J, Ward E, Ferlay J, Brawley O, et al. International variation in prostate cancer incidence and mortality rates. European urology. 2012;61(6):1079-92.

21. Chornokur G, Dalton K, Borysova ME, Kumar NB. Disparities at presentation, diagnosis, treatment, and survival in African American men, affected by prostate cancer. The Prostate. 2011;71(9):985-97.

22. Patel AR, Klein EA. Risk factors for prostate cancer. Nature Clinical Practice Urology. 2009;6(2):87-95.

23. Brawley OW. Prostate cancer epidemiology in the United States. World journal of urology. 2012;30(2):195-200.

24. Sirovich B, Gallagher PM, Wennberg DE, Fisher ES. Discretionary decision making by primary care physicians and the cost of US health care. Health Affairs. 2008;27(3):813-23.

25. Bostwick DG, Burke HB, Djakiew D, Euling S, Ho Sm, Landolph J, et al. Human prostate cancer risk factors. Cancer. 2004;101(S10):2371-490.

26. Kirby RS, Fitzpatrick JM, Irani J. Prostate cancer diagnosis in the new millennium: strengths and weaknesses of prostate‐specific antigen and the discovery and clinical evaluation of prostate cancer gene 3 (PCA3). BJU international. 2009;103(4):441-5.

27. CARVALHAL GF, SMITH DS, MAGER DE, RAMOS C, CATALONA WJ. Digital rectal examination for detecting prostate cancer at prostate specific antigen levels of 4 ng./ml. or less. The Journal of urology. 1999;161(3):835-9.

28. Lilja H, Ulmert D, Vickers AJ. Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nature Reviews Cancer. 2008;8(4):268-78.

29. Wolf A, Wender RC, Etzioni RB, Thompson IM, D'Amico AV, Volk RJ, et al. American Cancer Society guideline for the early detection of prostate cancer: update 2010. CA: a cancer journal for clinicians. 2010;60(2):70-98.

30. Eble JN. Pathology and genetics of tumours of the urinary system and male genital organs: Iarc; 2004.

31. Heidenreich A, Bellmunt J, Bolla M, Joniau S, Mason M, Matveev V, et al. EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and treatment of clinically localised disease. European urology. 2011;59(1):61-71.

32. Iczkowski KA, Lucia MS. Current perspectives on Gleason grading of prostate cancer. Current urology reports. 2011;12(3):216-22.

33. Lotan TL, Epstein JI. Clinical implications of changing definitions within the Gleason grading system. Nature Reviews Urology. 2010;7(3):136-42.

34. Epstein JI. An update of the Gleason grading system. The Journal of urology. 2010;183(2):433-40.

35. Cheng L, Montironi R, Bostwick DG, Lopez‐Beltran A, Berney DM. Staging of prostate cancer. Histopathology. 2012;60(1):87-117.

36. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Annals of surgical oncology. 2010;17(6):1471-4.

37. J Ramon, Denis L. (2007). Prostate Cancer. Springer, Berlin.

38. Edge S, Byrd D, Compton C. (2010) AJCC Cancer Staging Manual 7th Edition: Springer, New York.

39. Montie JE. Staging of prostate cancer. Current TNM classification and future prospects for prognostic factors. Cancer. 1995;75(S7):1814-8.

40. Falzarano SM, Magi-Galluzzi C. Prostate cancer staging and grading at radical prostatectomy over time. Advances in anatomic pathology. 2011;18(2):159-64.

41. Partin AW, Mangold LA, Lamm DM, Walsh PC, Epstein JI, Pearson JD. Contemporary update of prostate cancer staging nomograms (Partin Tables) for the new millennium. Urology. 2001;58(6):843-8.

42. Jani AB, Hellman S. Early prostate cancer: clinical decision-making. The Lancet. 2003;361(9362):1045-53.

43. Giberti C, Chiono L, Gallo F, Schenone M, Gastaldi E. Radical retropubic prostatectomy versus brachytherapy for low-risk prostatic cancer: a prospective study. World journal of urology. 2009;27(5):607-12.

44. Hammerer P, Madersbacher S. Landmarks in hormonal therapy for prostate cancer. BJU international. 2012;110(s1):23-9.

45. Oudard S. Progress in emerging therapies for advanced prostate cancer. Cancer treatment reviews. 2013;39(3):275-89.

46. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocrine reviews. 2004;25(2):276-308.

47. Chen FZ, Zhao XK. Prostate cancer: current treatment and prevention strategies. Iran Red Crescent Med J. 2013;15(4):279-84.

48. Damber JE, Aus G. Prostate cancer. Lancet. 2008;371(9625):1710-21.

49. Carles J, Castellano D, Climent MÁ, Maroto P, Medina R, Alcaraz A. Castration-resistant metastatic prostate cancer: current status and treatment possibilities. Clinical and Translational Oncology. 2012;14(3):169-76.

50. Felici A, Pino MS, Carlini P. A changing landscape in castration-resistant prostate cancer treatment. Frontiers in endocrinology. 2012;3:85.

51. Zong Y, Goldstein AS. Adaptation or selection—mechanisms of castration-resistant prostate cancer. Nature Reviews Urology. 2012;10(2):90-8.

52. Ahmed M, Li LC. Adaptation and clonal selection models of castration‐resistant prostate cancer: Current perspective. International Journal of Urology. 2013;20(4):362-71.

53. Sridhar SS, Freedland SJ, Gleave ME, Higano C, Mulders P, Parker C, et al. Castration-resistant prostate cancer: from new pathophysiology to new treatment. European urology. 2014;65(2):289-99.

54. Berthold DR, Pond GR, Soban F, de Wit R, Eisenberger M, Tannock IF. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study. Journal of Clinical Oncology. 2008;26(2):242-5.

55. Maluf FC, Smaletz O, Herchenhorn D. Castration-resistant prostate cancer: systemic therapy in 2012. Clinics. 2012;67(4):389-94.

56. Esteller M. Epigenetics in evolution and disease. The Lancet. 2008;372:S90-S6.

57. Esteller M. Epigenetics in cancer. The New England journal of medicine. 2008;358(11):1148-59.

58. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. Genes & development. 2009;23(7):781-3.

59. Flintoft L. Identical twins: epigenetics makes the difference. Nature Reviews Genetics. 2005;6(9):667-.

60. Payer B, Lee JT, Namekawa SH. X-inactivation and X-reactivation: epigenetic hallmarks of mammalian reproduction and pluripotent stem cells. Human genetics. 2011;130(2):265-80.

61. Ferguson-Smith AC. Genomic imprinting: the emergence of an epigenetic paradigm. Nature Reviews Genetics. 2011;12(8):565-75.

62. Sandoval J, Esteller M. Cancer epigenomics: beyond genomics. Current opinion in genetics & development. 2012;22(1):50-5.

63. Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. Carcinogenesis. 2010;31(1):27-36.

64. Portela A, Esteller M. Epigenetic modifications and human disease. Nature biotechnology. 2010;28(10):1057-68.

65. Kanwal R, Gupta S. Epigenetic modifications in cancer. Clinical genetics. 2012;81(4):303-11.

66. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nature Reviews Genetics. 2012;13(7):484-92.

67. Bird A. DNA methylation patterns and epigenetic memory. Genes & development. 2002;16(1):6-21.

68. Cedar H, Bergman Y. Programming of DNA methylation patterns. Annual review of biochemistry. 2012;81:97-117.

69. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nature Reviews Genetics. 2007;8(4):286-98.

70. Costa FF. Non-coding RNAs and new opportunities for the private sector. Drug discovery today. 2009;14(9):446-52.

71. Batista PJ, Chang HY. Long noncoding RNAs: cellular address codes in development and disease. Cell. 2013;152(6):1298-307.

72. Mattick JS, Makunin IV. Non-coding RNA. Human molecular genetics. 2006;15(suppl 1):R17-R29.

73. Costa FF. Non-coding RNAs, epigenetics and complexity. Gene. 2008;410(1):9-17.

74. Esteller M. Non-coding RNAs in human disease. Nature Reviews Genetics. 2011;12(12):861-74.

75. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. Annual review of medicine. 2009;60:167-79.

76. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO molecular medicine. 2012;4(3):143-59.

77. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science. 2007;318(5858):1931-4.

78. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. Journal of Clinical Oncology. 2009;27(34):5848-56.

79. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. Nature reviews Drug discovery. 2012;11(11):860-72.

80. Melo SA, Esteller M. Dysregulation of microRNAs in cancer: playing with fire. FEBS letters. 2011;585(13):2087-99.

81. Davalos V, Esteller M. MicroRNAs and cancer epigenetics: a macrorevolution. Current opinion in oncology. 2010;22(1):35-45.

82. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. Nature Reviews Cancer. 2006;6(4):259-69.

83. Kouzarides T. Chromatin modifications and their function. Cell. 2007;128(4):693-705.

84. Jenuwein T, Allis CD. Translating the histone code. Science. 2001;293(5532):1074-80.

85. Fullgrabe J, Kavanagh E, Joseph B. Histone onco-modifications. Oncogene. 2011;30(31):3391-403.

86. Cosgrove MS, Boeke JD, Wolberger C. Regulated nucleosome mobility and the histone code. Nature structural & molecular biology. 2004;11(11):1037-43.

87. Arrowsmith CH, Bountra C, Fish PV, Lee K, Schapira M. Epigenetic protein families: a new frontier for drug discovery. Nature reviews Drug discovery. 2012;11(5):384-400.

88. Falkenberg KJ, Johnstone RW. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. Nature reviews Drug discovery. 2014.

89. Dawson MA, Kouzarides T, Huntly BJ. Targeting epigenetic readers in cancer. The New England journal of medicine. 2012;367(7):647-57.

90. Musselman CA, Lalonde M-E, Côté J, Kutateladze TG. Perceiving the epigenetic landscape through histone readers. Nature structural & molecular biology. 2012;19(12):1218-27.

91. Zentner GE, Henikoff S. Regulation of nucleosome dynamics by histone modifications. Nature structural & molecular biology. 2013;20(3):259-66.

92. Waldmann T, Schneider R. Targeting histone modifications—Epigenetics in cancer. Current opinion in cell biology. 2013;25(2):184-9.

93. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. Cell research. 2011;21(3):381-95.

94. Miremadi A, Oestergaard MZ, Pharoah PD, Caldas C. Cancer genetics of epigenetic genes. Human molecular genetics. 2007;16(R1):R28-R49.

95. Mosammaparast N, Shi Y. Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. Annu Rev Biochem. 2010;79:155-79.

96. Kooistra SM, Helin K. Molecular mechanisms and potential functions of histone demethylases. Nature reviews Molecular cell biology. 2012;13(5):297-311.

97. Smith BC, Denu JM. Chemical mechanisms of histone lysine and arginine modifications. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms. 2009;1789(1):45-57.

98. Yang Y, Bedford MT. Protein arginine methyltransferases and cancer. Nature reviews Cancer. 2013;13(1):37-50.

99. Bianco-Miotto T, Chiam K, Buchanan G, Jindal S, Day TK, Thomas M, et al. Global levels of specific histone modifications and an epigenetic gene signature predict prostate cancer progression and development. Cancer Epidemiology Biomarkers & Prevention. 2010;19(10):2611-22.

100. Ellinger J, Kahl P, von der Gathen J, Rogenhofer S, Heukamp LC, Gütgemann I, et al. Global levels of histone modifications predict prostate cancer recurrence. The Prostate. 2010;70(1):61-9.

101. Seligson DB, Horvath S, McBrian MA, Mah V, Yu H, Tze S, et al. Global levels of histone modifications predict prognosis in different cancers. The American journal of pathology. 2009;174(5):1619-28.

102. Seligson DB, Horvath S, Shi T, Yu H, Tze S, Grunstein M, et al. Global histone modification patterns predict risk of prostate cancer recurrence. Nature. 2005;435(7046):1262-6.

103. Ke X-S, Qu Y, Rostad K, Li W-C, Lin B, Halvorsen OJ, et al. Genome-wide profiling of histone h3 lysine 4 and lysine 27 trimethylation reveals an epigenetic signature in prostate carcinogenesis. PloS one. 2009;4(3):e4687.

104. Hoffmann MJ, Engers R, Florl AR, Otte AP, Muller M, Schulz WA. Expression changes in EZH2, but not in BMI-1, SIRT1, DNMT1 or DNMT3B are associated with DNA methylation changes in prostate cancer. Cancer Biology and Therapy. 2007;6(9):1403.

105. Karanikolas BD, Figueiredo ML, Wu L. Comprehensive evaluation of the role of EZH2 in the growth, invasion, and aggression of a panel of prostate cancer cell lines. The Prostate. 2010;70(6):675-88.

106. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature. 2002;419(6907):624-9.

107. Yang YA, Yu J. EZH2, an epigenetic driver of prostate cancer. Protein & cell. 2013;4(5):331-41.

108. Bachmann IM, Halvorsen OJ, Collett K, Stefansson IM, Straume O, Haukaas SA, et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. Journal of Clinical Oncology. 2006;24(2):268-73.

109. Yu J, Yu J, Rhodes DR, Tomlins SA, Cao X, Chen G, et al. A polycomb repression signature in metastatic prostate cancer predicts cancer outcome. Cancer research. 2007;67(22):10657-63.

110. Chen Z, Wang L, Wang Q, Li W. Histone modifications and chromatin organization in prostate cancer. Epigenomics. 2010;2(4):551-60.

111. Chin SP, Dickinson JL, Holloway AF. Epigenetic regulation of prostate cancer. Clinical epigenetics. 2011;2(2):151-69.

112. Hayami S, Kelly JD, Cho HS, Yoshimatsu M, Unoki M, Tsunoda T, et al. Overexpression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers. International Journal of Cancer. 2011;128(3):574-86.

113. Kahl P, Gullotti L, Heukamp LC, Wolf S, Friedrichs N, Vorreuther R, et al. Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of prostate cancer recurrence. Cancer research. 2006;66(23):11341-7.

114. Opel M, Lando D, Bonilla C, Trewick SC, Boukaba A, Walfridsson J, et al. Genome-wide studies of histone demethylation catalysed by the fission yeast homologues of mammalian LSD1. PloS one. 2007;2(4):e386.

115. Yoshimatsu M, Toyokawa G, Hayami S, Unoki M, Tsunoda T, Field HI, et al. Dysregulation of PRMT1 and PRMT6, Type I arginine methyltransferases, is involved in various types of human cancers. International Journal of Cancer. 2011;128(3):562-73.

116. Carvalho JR, Filipe L, Costa VL, Ribeiro FR, Martins AT, Teixeira MR, et al. Detailed analysis of expression and promoter methylation status of apoptosis-related genes in prostate cancer. Apoptosis. 2010;15(8):956-65.

117. Hyllus D, Stein C, Schnabel K, Schiltz E, Imhof A, Dou Y, et al. PRMT6-mediated methylation of R2 in histone H3 antagonizes H3 K4 trimethylation. Genes & development. 2007;21(24):3369-80.

118. Guccione E, Bassi C, Casadio F, Martinato F, Cesaroni M, Schuchlautz H, et al. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. Nature. 2007;449(7164):933-7.

119. Kirmizis A, Santos-Rosa H, Penkett CJ, Singer MA, Vermeulen M, Mann M, et al. Arginine methylation at histone H3R2 controls deposition of H3K4 trimethylation. Nature. 2007;449(7164):928-32.

120. Vieira FQ, Costa-Pinheiro P, Ramalho-Carvalho J, Pereira A, Menezes FD, Antunes L, et al. Deregulated expression of selected histone methylases and demethylases in prostate carcinoma. Endocrine-related cancer. 2014;21(1):51-61.

121. Neault M, Mallette FA, Vogel G, Michaud-Levesque J, Richard S. Ablation of PRMT6 reveals a role as a negative transcriptional regulator of the p53 tumor suppressor. Nucleic acids research. 2012;40(19):9513-21.

122. Stein C, Riedl S, Ruthnick D, Notzold RR, Bauer UM. The arginine methyltransferase PRMT6 regulates cell proliferation and senescence through transcriptional repression of tumor suppressor genes. Nucleic acids research. 2012;40(19):9522-33.

123. Kleinschmidt MA, de Graaf P, van Teeffelen HA, Timmers HT. Cell cycle regulation by the PRMT6 arginine methyltransferase through repression of cyclin-dependent kinase inhibitors. PloS one. 2012;7(8):e41446.

124. Bitting RL, Armstrong AJ. Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. Endocrine-related cancer. 2013;20(3):R83-99.

125. Carver BS. Strategies for targeting the androgen receptor axis in prostate cancer. Drug Discov Today. 2014;19(9):1493-7.

126. Tai S, Sun Y, Squires JM, Zhang H, Oh WK, Liang CZ, et al. PC3 is a cell line characteristic of prostatic small cell carcinoma. The Prostate. 2011;71(15):1668-79.

127. Frankel A, Yadav N, Lee J, Branscombe TL, Clarke S, Bedford MT. The novel human protein arginine N-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity. The Journal of biological chemistry. 2002;277(5):3537-43.

128. Boulanger MC, Liang C, Russell RS, Lin R, Bedford MT, Wainberg MA, et al. Methylation of Tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression. Journal of virology. 2005;79(1):124-31.

129. El-Andaloussi N, Valovka T, Toueille M, Steinacher R, Focke F, Gehrig P, et al. Arginine methylation regulates DNA polymerase beta. Molecular cell. 2006;22(1):51-62.

130. Wang P, Ma Q, Luo J, Liu B, Tan F, Zhang Z, et al. Nkx3.1 and p27(KIP1) cooperate in proliferation inhibition and apoptosis induction in human androgen-independent prostate cancer cells. Cancer investigation. 2009;27(4):369-75.

131. Cote RJ, Shi Y, Groshen S, Feng AC, Cordon-Cardo C, Skinner D, et al. Association of p27Kip1 levels with recurrence and survival in patients with stage C prostate carcinoma. Journal of the National Cancer Institute. 1998;90(12):916-20.

132. Tsihlias J, Kapusta LR, DeBoer G, Morava-Protzner I, Zbieranowski I, Bhattacharya N, et al. Loss of cyclin-dependent kinase inhibitor p27Kip1 is a novel

prognostic factor in localized human prostate adenocarcinoma. Cancer Res. 1998;58(3):542-8.

133. Yang RM, Naitoh J, Murphy M, Wang HJ, Phillipson J, deKernion JB, et al. Low p27 expression predicts poor disease-free survival in patients with prostate cancer. The Journal of urology. 1998;159(3):941-5.

134. Cordon-Cardo C, Koff A, Drobnjak M, Capodieci P, Osman I, Millard SS, et al. Distinct altered patterns of p27KIP1 gene expression in benign prostatic hyperplasia and prostatic carcinoma. Journal of the National Cancer Institute. 1998;90(17):1284-91.

135. Brehmer B, Biesterfeld S, Jakse G. Expression of matrix metalloproteinases (MMP-2 and -9) and their inhibitors (TIMP-1 and -2) in prostate cancer tissue. Prostate cancer and prostatic diseases. 2003;6(3):217-22.

136. Bachmeier BE, Nerlich AG, Lichtinghagen R, Sommerhoff CP. Matrix metalloproteinases (MMPs) in breast cancer cell lines of different tumorigenicity. Anticancer research. 2001;21(6A):3821-8.

137. Karakiulakis G, Papanikolaou C, Jankovic SM, Aletras A, Papakonstantinou E, Vretou E, et al. Increased type IV collagen-degrading activity in metastases originating from primary tumors of the human colon. Invasion & metastasis. 1997;17(3):158-68.

138. Pagenstecher A, Wussler EM, Opdenakker G, Volk B, Campbell IL. Distinct expression patterns and levels of enzymatic activity of matrix metalloproteinases and their inhibitors in primary brain tumors. Journal of neuropathology and experimental neurology. 2001;60(6):598-612.

139. Iczkowski KA. Cell adhesion molecule CD44: its functional roles in prostate cancer. American journal of translational research. 2010;3(1):1-7.

140. Lesley J, Hyman R, Kincade PW. CD44 and its interaction with extracellular matrix. Advances in immunology. 1993;54:271-335.

141. Underhill C. CD44: the hyaluronan receptor. Journal of cell science. 1992;103 (Pt 2):293-8.

142. Iczkowski K, Pantazis C, Collins J. The loss of expression of CD44 standard and variant isoforms is related to prostatic carcinoma development and tumor progression. JOURNAL OF UROLOGIC PATHOLOGY. 1997;6:119-30.

143. Nagabhushan M, Pretlow TG, Guo YJ, Amini SB, Pretlow TP, Sy MS. Altered expression of CD44 in human prostate cancer during progression. American journal of clinical pathology. 1996;106(5):647-51.

144. De Marzo AM, Bradshaw C, Sauvageot J, Epstein JI, Miller GJ. CD44 and CD44v6 downregulation in clinical prostatic carcinoma: relation to Gleason grade and cytoarchitecture. The Prostate. 1998;34(3):162-8.

145. Kallakury BV, Yang F, Figge J, Smith KE, Kausik SJ, Tacy NJ, et al. Decreased levels of CD44 protein and mRNA in prostate carcinoma. Correlation with tumor grade and ploidy. Cancer. 1996;78(7):1461-9.

146. Noordzij MA, van Steenbrugge GJ, Verkaik NS, Schroder FH, van der Kwast TH. The prognostic value of CD44 isoforms in prostate cancer patients treated by radical prostatectomy. Clinical cancer research : an official journal of the American Association for Cancer Research. 1997;3(5):805-15.

147. Verkaik NS, van Steenbrugge GJ, van Weerden WM, Bussemakers MJ, van der Kwast TH. Silencing of CD44 expression in prostate cancer by hypermethylation of the CD44 promoter region. Laboratory investigation; a journal of technical methods and pathology. 2000;80(8):1291-8.

148. Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. Cancer cell. 2010;18(1):11-22.

149. da Silva HB, Amaral EP, Nolasco EL, de Victo NC, Atique R, Jank CC, et al. Dissecting major signaling pathways throughout the development of prostate cancer. Prostate cancer. 2013;2013.

150. Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. Pten is essential for embryonic development and tumour suppression. Nature genetics. 1998;19(4):348-55.

151. Morgan TM, Koreckij TD, Corey E. Targeted therapy for advanced prostate cancer: inhibition of the PI3K/Akt/mTOR pathway. Current cancer drug targets. 2009;9(2):237-49.

152. Kreisberg JI, Malik SN, Prihoda TJ, Bedolla RG, Troyer DA, Kreisberg S, et al. Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. Cancer Res. 2004;64(15):5232-6.

153. Ayala G, Thompson T, Yang G, Frolov A, Li R, Scardino P, et al. High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10(19):6572-8.

154. Bedolla R, Prihoda TJ, Kreisberg JI, Malik SN, Krishnegowda NK, Troyer DA, et al. Determining risk of biochemical recurrence in prostate cancer by immunohistochemical detection of PTEN expression and Akt activation. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(13):3860-7.

155. Skvortsova I, Skvortsov S, Stasyk T, Raju U, Popper BA, Schiestl B, et al. Intracellular signaling pathways regulating radioresistance of human prostate carcinoma cells. Proteomics. 2008;8(21):4521-33.

156. Grunwald V, DeGraffenried L, Russel D, Friedrichs WE, Ray RB, Hidalgo M. Inhibitors of mTOR reverse doxorubicin resistance conferred by PTEN status in prostate cancer cells. Cancer Res. 2002;62(21):6141-5.

157. Qian DZ, Rademacher BL, Pittsenbarger J, Huang CY, Myrthue A, Higano CS, et al. CCL2 is induced by chemotherapy and protects prostate cancer cells from docetaxel-induced cytotoxicity. The Prostate. 2010;70(4):433-42.

158. Lonergan PE, Tindall DJ. Androgen receptor signaling in prostate cancer development and progression. Journal of carcinogenesis. 2011;10:20.

159. Kinoshita H, Shi Y, Sandefur C, Meisner LF, Chang C, Choon A, et al. Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer. Cancer Res. 2000;60(13):3623-30.

160. Nakayama T, Watanabe M, Suzuki H, Toyota M, Sekita N, Hirokawa Y, et al. Epigenetic regulation of androgen receptor gene expression in human prostate cancers. Laboratory investigation; a journal of technical methods and pathology. 2000;80(12):1789-96.

161. Bonaccorsi L, Carloni V, Muratori M, Salvadori A, Giannini A, Carini M, et al. Androgen receptor expression in prostate carcinoma cells suppresses alpha6beta4 integrin-mediated invasive phenotype. Endocrinology. 2000;141(9):3172-82.

162. Cinar B, Koeneman KS, Edlund M, Prins GS, Zhau HE, Chung LW. Androgen receptor mediates the reduced tumor growth, enhanced androgen responsiveness, and selected target gene transactivation in a human prostate cancer cell line. Cancer Res. 2001;61(19):7310-7.